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(54) Title: REGULATION OF bcl-2 GENE EXPRESSION

(57) Abstract

   The present invention provides novel anticode oligomers and methods of using them for controlling the growth of cancer cells expressing the bcl-2 gene.

REGULATION OF bcl-2 GENE EXPRESSION

Reference to Government Grants

The research in this pat nt application was supported in part by National Institutes of Health grant CA 26380. The United States government has certain rights in the invention.

Field of the Invention

The present invention relates to the field of treatments for cancer and more particularly to the field of anticode oligomer treatments for cancer.

Related Application Data

This application is a continuation-in-part of Serial No. 07/840,716 filed February 21, 1992, which was a continuation in part of Serial No. 07,288,692 filed December 22, 1988, which has been abandoned.

Background of the Invention

Current approaches to cancer treatment suffer from a lack of specificity. The majority of drugs that have been developed are natural products or derivatives that either block enzyme pathways or randomly interact with DNA. Due to low therapeutic indices, most cancer treatment drugs are accompanied by serious dose-limiting toxicities. The administration of drugs to treat cancer kills not only cancer cells but also normal non-cancerous cells. Because of these deleterious effects, treatments that are more specific for cancerous cells are needed.

It has been found that a class of genes, the oncogenes, plays a large role in the transformation and maintenance of the cancerous state and that turning off these genes, or otherwise inhibiting their effects, can return a cell to a normal phenotype. The role of oncogenes in the etiology of many human cancers has been

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reviewed in Bishop, "Cellular Oncogenes and Retroviruses," *Science*, 235:305-311 (1987). In many types of human tumors, including lymphomas and leukemias, the human bcl-2 gene is overexpressed, and may be
5   associated with tumorigenicity (Tsujimoto et al. Involvement of the bcl-2 gene in human follicular lymphoma, Science 228:1440-1443 (1985)).

        Antisense oligodeoxynucleotides are one example
10   of a specific therapeutic tool with the potential for ablating oncogene function. These short (usually about 30 bases) single-stranded synthetic DNAs have a complementary base sequence to the target mRNA and form a hybrid duplex by hydrogen bonded base pairing. This
15   hybridization can be expected to prevent expression of the target mRNA code into its protein product and thus preclude subsequent effects of the protein product. Because the mRNA sequence expressed by the gene is termed the sense sequence, the complementary sequence is termed
20   the antisense sequence. Under some circumstances, inhibition of mRNA would be more efficient than inhibition of an enzyme's active site, since one mRNA molecule gives rise to multiple protein copies.

        Synthetic oligodeoxynucleotides complementary
25   to (antisense) mRNA of the *c-myc* oncogene have been used to specifically inhibit production of *c-myc* protein, thus arresting the growth of human leukemic cells *in vitro*, Holt et al., *Mol. Cell Biol.* 8:963-973 (1988), and Wickstrom et al., *Proc. Natl. Acad. Sci.* USA,
30   85:1028-1-32 (1988). Oligodeoxynucleotides have also been employed as specific inhibitors of retroviruses, including the human immunodeficiency virus (HIV-I), Zamecnik and Stephenson, *Proc. Natl. Acad. Sci.* USA, 75:280-284 (1978) and Zamecnik et al., *Proc. Natl. Acad.*
35   *Sci.* USA, 83:4143-4146 (1986).

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Summary of the Invention

The invention provides anticode oligomers and methods for inhibiting growth of cancer cells. The growth of lymphoma or leukemia cells, which are types of lymphocytes, are inhibitied by the anticode oligomers and methods of the invention. An anticode oligomer complementary to at least an effective portion of the mRNA sense strand to the human *bcl*-2 gene is provided and cells are then contacted with the anticode oligomer in a concentration sufficient to inhibit growth of the cells. The methods of the invention are suitable for inhibiting growth of lymphoma/leukemia cells that express the human *bcl*-2 gene and have a t (14; 18) chromosomal translocation as well as those that express the *bcl*-2 gene but do not have a t (14; 18) chromosomal translocation.

In accordance with preferred embodiments, the anticode oligomer is substantially complementary to a strategic site in the pre-mRNA sense strand or substantially complementary to the mRNA. A preferred strategic site is the translation-initiation site of the pre-mRNA coding strand. Alternative strategic sites include coding sites for splicing, transport or degradation. The subject anticode oligomer either in its "native," unmodified form -- oligonucleotide -- or as a derivative, is brought into contact with the target lymphoma or leukemia cells. For *in vivo* therapeutic use, a derivative of the "native" oligonucleotide, such as the phosphorothioate form is preferable since it is believed that these forms are more resistant to degradation, notwithstanding the fact that response times to some analogues, such as the phosphorothioate analogs, has been found to be somewhat slower than to the "native" form of the oligonucleotide.

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A preferred anticode oligomer, denominated
herein the TI-AS (translation initiation antic de
oligomer) is an oligodeoxynucleotide which straddles the
translation-initiation site of the mRNA coding strand of

5   the human bcl-2 gene and is complementary to this region.
More preferably, this nucleotide comprises a TAC portion
which is complementary to the ATG initiation sequence of
the coding strand for the bcl-2 gene, and preferably
further comprises flanking portions of two to about one

10   hundred bases, more preferably from about five to about
twenty bases, which are complementary to portions of the
bcl-2 gene coding strand flanking said initiation
sequence. The TI-AS nucleotide has been found effective
at inhibiting the growth of the target cells both in the

15   presence and absence of serum.

Alternatively, the anticode oligomer comprises
an antisense nucleotide complementary to at least an
effective portion of the splice donor site of the pre-
mRNA coding strand for the human bcl-2 gene. More

20   particularly, this nucleotide comprises a CA portion
which is complementary to the GT splice donor of the
bcl-2, and again comprises flanking portions of two to
about one hundred bases, preferably from about five to
about twenty bases, which are complementary to portions

25   of the bcl-2 gene coding strand flanking said splice
donor.

In yet another embodiment, the anticode
oligomer is complementary to at least an effective
portion of the splice acceptor region of the pre-mRNA

30   coding strand for the human bcl-2 gene. This oligomer
comprises at least a TC portion which is complementary to
the AG splice acceptor of the bcl-2 gene, and again
comprises flanking portions of two to about one hundred,
preferably from about five to about twenty bases which

35   are complementary to portions of the bcl-2 gene coding

strand flanking said acceptor. The subject oligomer may
also be selected to overlap the coding site for the 26
kDa protein, *bcl*-2-alpha or for the 22 kDa protein,
bcl-2-beta, protein products of the *bcl*-2 gene.
Preferably the oligomer is selected to minimize homology
with anticode oligomers for pre-mRNA or mRNA coding
strands for other gene sequences.

Accordingly, a primary object of the present
invention is the provision of novel anticode oligomers,
which are useful in inhibiting the growth of cancer
cells. The present invention also includes compositions
for inhibiting the growth of tumor cells, which
compositions comprise the anticode oligomer of the
present invention together with a pharmaceutically
acceptable carrier.

A further object of the present invention is
the provision of methods for inhibiting the growth of
cancer cells using said anticode oligomers. As a feature
of the present invention, it was discovered that average
reductions of 30-40% in the relative levels of bcl-2
protein markedly enhanced the sensitivity of lymphoma
cells, in particular, t(14;18)-containing lymphoma cell
lines to cancer chemotherapeutic agents, including
conventional anticancer drugs. Such reductions were
achieved by introducing into tumor cells an anticode
oligomer which binds to either pre-mRNA or mRNA expressed
from the bcl-2 gene. Two methods were used in the
present invention to introduce said anticode oligomers to
tumor cells. One method involved contacting the tumor
cells with a composition comprising the anticode
oligomers. Another method involved transfecting the
tumor cells with a vector encoding an antisense
oligonucleotide. Introducing an anticode oligomer to
tumor cells achieved a reduction of bcl-2 expression and

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increases the chemosensitivity of neoplastic c lls to cancer chemotherapeutic agents or anticancer drugs.

Accordingly, the present invention achieved a method of killing tumor cells by introducing to tumor

5   cells anticode oligomers which reduce *bcl-2* gene expression or impair Bcl-2 protein function before contacting the cells with cancer chemotherapeutic agents. The cancer chemotherapeutic agents reduced the numbers of viable malignant cells, and the portion of tumor cells

10   killed was greater than the portion which would have been killed by the same amount of drug in the absence of introducing the anticode oligomer oligodeoxynucleotide to the cells.

15   These and other objects of the present invention will become apparent from the following detailed description.

Brief Description of the Drawings

Figures 1 (a - d) show graphs of the effects of

20   varying concentrations of antisense oligodeoxynucleotides on inhibition of cell proliferation.

Figures 2(a) and (b) show graphs of the concentration dependence of inhibition of cell proliferation by antisense normal and phosphorothioate

25   oligodeoxynucleotides. Oligodeoxynucleotide additions to cultures included TI-AS phosphorothioate (o and o; two separate experiments), TI-S phosphorothioate (Δ), TI-AS normal (□), and TI-S normal (Δ).

Figure 3 shows the results of gel

30   electrophoresis of six antisense oligonucleotides targeted against the translation initiation site of *bcl-2* mRNA.

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Figures 4(a) and (b) show the degree of DNA fragmentation resulting from oligonucleotide treatment of RS11846 cells. Figure 4(a) shows the effect of oligonucleotides targeted against the translation initiation site. Figure 4(b) shows the effect of oligonucleotides directed against the 5'-cap region of *bcl*-2 mRNA.

Figure 5 is a graph showing the concentration-dependence of inhibition by an antisense oligonucleotide targeted against the translation initiation site of *bcl*-2 mRNA.

Figures 6(a) and (b) are graphs showing the results of immunofluorescence analysis of *bcl*-2 protein levels in oligonucleotide-treated cells.

Figures 7(a-d) are FACS profiles for 697 cells before and after treatment with *bcl*-2 antisense oligonucleotides.

Figure 8 (a-c) show bcl-2 antisense oligodeoxynucleotides producing sequence-specific reductions in bcl-2 mRNA and bcl-2 protein and producing increased sensitivity of SU-DHL-4 cells to cancer chemotherapeutic drugs.

Figures 9(a) and (b) demonstrate the differential effects of bcl-2 antisense oligomers on chemosensitivity of 32D-bcl-2 and 32D-BHRF-1 cells.

Figures 10 (a-b) show reduction of chemoresistance of RS11846 cells from inducible bcl-2 antisense expression from an expression plasmid.

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Figure 11 shows
methylph sphonate/phosphodiester bcl-2 antisense
olig mers inducing death of DOHH2 lymphoma cells.

Figure 12 shows methylphosphonate
(MP)/Phosphodiester (PO) chimeric oligomers inhibiting
growth of MCF-7 human breast cancer cells.

Figures 13(a-c)) show optimization of antisense
bcl-2 oligomer sequences.

Detailed Description of the Invention
        According to the invention, anticode oligomers
are provided for inhibiting cancer cell growth, for
increasing the sensitivity of cancer cells to cancer
chemotherapeutic agents, or for inducing cancer cell
death alone or in combination with any one or more cancer
chemotherapeutic agents.

Definitions
        As used herein, the term "anticode oligomers"
means anticode oligonucleotides and analogs thereof and
refers to a range of chemical species that recognize
polynucleotide target sequences through hydrogen bonding
interactions with the nucleotide bases of the target
sequences. The target sequences may be single- or
double-stranded RNA or single- or double-stranded DNA.

        The anticode oligonucleotides and analogs
thereof may be RNA or DNA, or analogs of RNA or DNA,
commonly referred to as antisense oligomers or antisense
oligonucleotides. Such RNA or DNA analogs comprise but
are not limited to 2-'O-alkyl sugar modifications,
methylphosphonate, phosphorothioate, phosphordithioate,
formacetal, 3'-thioformacetal, sulfone, sulfamate, and
nitroxide backbone modifications, and analogs wherein the
base moieties have been modified. In addition, analogs

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of oligomers may be polym rs in which the sugar moiety
has been modified or replaced by another suitable moiety,
resulting in polymers which include, but are not limited
to, morpholino analogs and peptide nucleic acid (PNA)

5   analogs (Egholm, et al. Peptide Nucleic Acids (PNA) -
Oligonucleotide Analogues with an Achiral Peptide
Backbone, (1992)).

Anticode analogs may also be mixtures of any of
the oligonucleotide analog types together or in

10   combination with native DNA or RNA. At the same time,
the oligonucleotides and analogs thereof may be used
alone or in combination with one or more additional
oliognucleotides or analogs thereof. The
oligonucleotides may be from about 10 to about 1,000

15   nucleotides long. Although oliogonucleotides of 10 to
100 nucleotides are useful in the invention, preferred
oligonucleotides range from about 15 to about 24 bases in
length.

Anticode oligonucleotides and analogs thereof

20   also comprise conjugates of the oligonucleotides and
analogs thereof. (John Goodchild, Congugates of
Oligonucleotides and Modified Oligonucleotides: A Review
of Their Synthesis and Properties, Bioconjugate
Chemistry, Volume 1 No. 3, May/June (1990)). Such

25   conjugates having properties to improve the uptake,
pharmacokinetics, and nuclease resistance of the
oligonucleotide, or the ability to enhance cross-linking
or cleavage of the target sequence by the
oligonucleotide.

30   As used herein, the term "cell proliferation"
refers to cell division rate/cell cycle. The term
"growth," as used herein, encompasses both increased cell
numbers due to faster cell division and due to slower
rates of cell death.

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As used herein, bcl-2 gene expression refers to
bcl-2 protein production from the human bcl-2 gene; e.g.
reduced bcl-2 gene expression means reduced levels of
bcl-2 protein.

5   As used herein, "strategic sites" are defined
as any site which when bound by the claimed anticode
molecules or analogs thereof results in inhibiting
expression of the bcl-2 gene.

         As used herein, the term "sequence portion" is
10   a portion of the nucleotide sequence of an RNA
oligonucleotide. In appropriate contexts, "sequence
portion" may refer to a portion of the nucleotide
sequence of a DNA segment or DNA oligonucleotide.

         Uncontrolled cell proliferation is a marker for
15   a cancerous or abnormal cell type. Normal, non-cancerous
cells divide regularly, at a frequency characteristic for
the particular type of cell. When a cell has been
transformed into a cancerous state, the cell divides and
proliferates uncontrollably. Inhibition of proliferation
20   modulates the uncontrolled division of the cell.
Containment of cell division often correlates with a
return to a non-cancerous state.

         A human gene termed *bcl-2* (B cell
lymphoma/leukemia-2) is implicated in the etiology of
25   some common lymphoid tumors, Croce et al., "Molecular
Basis Of Human B and T Cell Neoplasia," in: *Advance in
Viral Oncology*, 7:35-51, G. Klein (ed.), New York: Raven
Press, 1987. High levels of expression of the human
bcl-2 gene have been found in all lymphomas with t (14;
30   18) chromosomal translocations including most follicular
B cell lymphomas and many large cell non-Hodgkin's
lymphomas. High levels of expression of the *bcl-2* gene
have also been found in certain leukemias that do not

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have a t(14; 18) chromosomal translocation, including
most cases of chronic lymphocytic leukemia acute, many
lymphocytic leuk mias of the pre-B cell type,
neuroblastomas, nasophryngeal carcinomas, and many
5   adenocarcinomas of the prostate, breast, and colon.
(Reed et al., Differential expression of bcl-2
protooncogene in neuroblastoma and other human tumor cell
lines of neural origin. Cancer Res. 51:6529 (1991); Yunis
et al. Bcl-2 and other genomic alterations in the
10   prognosis of large-cell lymphomas. New England J. Med.
320:1047; Campos et al. High expression of bcl-2 protein
in acute myeloid leukemia is associated with poor
response to chemotherapy. Blood 81:3091-3096 (1993);
McDonnell et al. Expression of the protooncogene bcl-2
15   and its association with emergence of androgen-
independent prostate cancer. Cancer Res. 52:6940-6944
(1992);   Lu Q-L, et al. Bcl-2 protooncogene expression
in Epstein Barr Virus-Associated Nasopharyngeal
Carcinoma, Int. J Cancer 53:29-35 (1993); Bonner et al.
20   bcl-2 protooncogene and the gastrointestinal mucosal
epithelial tumor progression model as related to proposed
morphologic and molecular sequences, Lab Invest. 68:43A
(1993)).

       While not limited to the following explanation,
25   the present invention exploits cellular mechanisms
concerned with normal cell death. Because most types of
cells have a finite life span and are programmed to die,
uncontrollable cell accumulation can also result because
of a defect in normal cell death mechanisms rather than
30   through an increased rate of cell division. The bcl-2
gene contributes to the pathogenesis of cancer primarily
by prolonging cell survival rather than accelerating cell
division.

       Antisense oligomers suitable for use in the
35   invention include nucleotide oligomers which are two to

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two hundred nucleotide bases long; more preferably ten to
forty bases long; most preferably twenty bases long. The
oligonucleotides are preferably selected from those
oligonucleotides complementary to strategic sites along

5   the pre-mRNA of *bcl*-2, such as the translation initiation
site, donor and splicing sites, or sites for
transportation or degradation.

Blocking translation at such strategic sites
prevents formation of a functional *bcl*-2 gene product.

10   It should be appreciated, however, that any combination
or subcombination of anticode oligomers, including
oliognucleotides complementary or substantially
complementary to the *bcl*-2 pre-mRNA or mRNA that inhibit
cell proliferation is suitable for use in the invention.

15   For example, oligodeoxynucleotides complementary to
sequence portions of contiguous or non-contiguous
stretches of the *bcl*-2 RNA may inhibit cell proliferation
and would thus be suitable for use in the invention.

It should also be appreciated that anticode

20   oligomers suitable for use in the invention may also
include oligonucleotides flanking those complementary or
substantially complementary to such sequence portions as
the strategic or other sites along the *bcl*-2 mRNA. The
flanking sequence portions are preferably from two to

25   about one hundred bases, more preferably from about five
to about twenty bases in length. It is also preferable
that the anticode oligomers be complementary to a
sequence portion of the pre-mRNA or mRNA that is not
commonly found in pre-mRNA or mRNA of other genes to

30   minimize homology of anticode oligomers for pre-mRNA or
mRNA coding strands from other genes.

Preferred antisense, or complementary,
olig deoxynucleotides are listed in Table 1.

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TABLE I
bcl-2 Oligodeoxynucleotides

translation initiation
antisense (TI-AS)   3'...CCCTTCCTACCGCGTGCGAC...5'

5   bcl-2   5'...CTTTTCCTCTGGGAAGGATGGCGCACGCTGGGAGA...3'

splice donor
antisense (SD-AS)   31...CCTCCGACCCATCCACGTAG...5'

bcl-2   5'...ACGGGGTAC...GGAGGCTGGGTAGGTGCATCTGGT...3'

splice acceptor
10 antisense (SA-AS)   3'...GTTGACGTCCTACGGAAACA...5'

bcl-2   5'...CCCCCAACTGCAGGATGCCTTTGTGGAACTGTACGG...3'

It will be appreciated by those skilled in the
art to which this invention pertains, that anticode
oligomers having a greater or lesser number of
15 substituent nucleotides, or that extend further along the
bcl-2 mRNA in either the 3' or 5' direction than the
preferred embodiments, but which also inhibit cell
proliferation are also within the scope of the invention.

It is preferable to use chemically modified
20 derivatives or analogs of anticode oligomers in the
performance of the invention rather than "native" or
unmodified oligodeoxynucleotides. "Native"
oligodeoxynucleotides can be conveniently synthesized
with a DNA synthesizer using standard phosphoramidite
25 chemistry. Suitable derivatives, and methods for
preparing the derivatives, include phosphorothioate,
Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988);
methylphosphonate, Blake et al., *Biochemistry*
24:6132-6138 (1985) and alphadeoxynucleotides, Morvan et
30 al., *Nucl. Acids Res.*. 14:5019-5032 (1986), 2'-O-methyl-
ribonucleosides (Monia et al. Evaluation of 2'-modified
oligonucleotides containing 2' deoxy gaps as antisense
inhibitors of gene expresssion. <u>J. Biol. Chem</u>. 268:14514-
14522 (1933)), and covalently-linked derivatives such as

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acridine, Asseline et al., *Proc. Natl Acad. Sci.* USA
81:3297-3201 (1984); alkylated (e.g.,
N-2-chlorocethylamine), Knorre et al., *Biochemie*
67:783-789 (1985) and Vlassov et al., *Nucl. Acids Res.*
14:4065-4076 (1986); phenazine, Knorre et al., *supra*, and
Vlassov et al., *supra*; 5-methyl-N^4-N^4-ethanocytosine, Webb
et al., *Nucl. Acids Res.* 14:7661-7674 (1986);
Fe-ethylenediamine tetraacetic acid (EDTA) and analogues,
Boutorin et al., *FEBS Letter's* 172:43-46 (1984);
5-glycylamido-1, 10-o-phenanthroline, Chi-Hong et al.,
Proc. Natl. Acad. Sci. USA 83:7147-7151 (1986); and
diethylenetriaamine-pentaacetic acid (DTPA) derivatives,
Chu et al., *Proc. Natl. Acad. Sci.* 82:963-967 (1985).
All of the above publications are hereby specifically
incorporated by reference as if fully set forth herein.

The anticode oligomer of the present invention
can also be combined with a pharmaceutically acceptable
carrier for administration to a subject or for ex-vivo
administration. Examples of suitable pharmaceutical
carriers are a variety of cationic lipids, including, but
not limited to N-(1-2,3-dioleyloxy)propyl)-
n,n,n-trimethylammonium chloride (DOTMA) and
dioleoylphophotidylethanolamine (DOPE)]. Liposomes are
also suitable carriers for the anticode oligomers of the
invention.

The anticode oligomers may be administered to
patients by any effective route, including intravenous,
intramuscular, intrathecal, intranasal, intraperitoneal,
subcutaneous injection, *in situ* injection and oral
administration. Oral administration requires enteric
coatings to protect the claimed anticode molecules and
analogs thereof from degradation along the
gastrointestinal tract. The anticode oligomers may be
mixed with an amount of a physiologically acceptable
carrier or diluent, such as a saline solution or other

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suitable liquid. The anticode oligomers may also be
combined with liposomes or other carrier means to
protect the anticode molecules or analogs th reof from
degradation until they reach their targets and/or
facilitate movement of the anticode molecules or analogs
thereof across tissue barriers.

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        The anticode oligomers may also be useful for
ex vivo bone marrow purging. Normally, the amounts of
conventional cancer chemotherapeutic agents or drugs and
irradiation that a patient can receive are limited by
toxicity to the marrow, i.e., anemia (fatigue, heart
failure), thrombocytopenia (bleeding), neutropenia
(infection). Thus, in order to deliver sufficient
concentrations of drugs and irradiation to totally
eradicate the tumor, the physician would simultaneously
destroy the patient's normal bone marrow cells leading to
patient demise. Alternatively, large amounts of bone
marrow can be surgically extracted from the patient and
stored in vitro. while the patient receives aggressive
conventional treatment. The patient can then be rescued
by reinfusion of their own bone marrow cells, but only if
that marrow has been "purged" of residual malignant
cells. The claimed anticode oligomers could be used to
remove residual malignant cells from the bone marrow.

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        The anticode oligomers are administered in
amounts effective to inhibit cancer or neoplastic cell
growth. The actual amount of any particular anticode
oligomer administered will depend on factors such as the
type of cancer, the toxicity of the anticode oligomer to
other cells of the body, its rate of uptake by cancer
cells, and the weight and age of the individual to whom
the anticode oligomer is administered. Because of
inhibitors present in human serum that may interfere with
the action of the anticode oligomer an effective amount
of the anticode oligomer for each individual may vary.

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An effective dosage for the patient can be ascertained by conventional methods such as incrementally increasing the dosage of the anticode olig mer from an amount ineffective to inhibit cell proliferation to an effective amount. It is expected that concentrations presented to cancer cells in the range of about 0.001 micromolar to about 100 micromolar will be effective to inhibit cell proliferation.

The anticode oligomers are administered to the patient for at least a time sufficient to inhibit proliferation of the cancer cells. The anticode oligomers are preferably administered to patients at a frequency sufficient to maintain the level of anticode oligomers at an effective level in or around the cancer cells. To maintain an effective level, it may be necessary to administer the anticode oligomers several times a day, daily or at less frequent intervals. Anticode oligomers are administered until cancer cells can no longer be detected, or have been reduced in number such that further treatment provides no significant reduction in number, or the cells have been reduced to a number manageable by surgery or other treatments. The length of time that the anticode oligomers are administered will depend on factors such as the rate of uptake of the particular oligodeoxynucleotide by cancer cells and time needed for the cells to respond to the oligodeoxynucleotide. *In vitro*, maximal inhibition of neoplastic cell growth by "native," unmodified anticode oligomers occurred two days after initiation of cultures,, whereas phosphorothioate oligodeoxynucleotides required 4 to 7 days to achieve maximal inhibition. *In vivo*, the time necessary for maximal inhibition of cell proliferation may be shorter or longer.

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The anticode oligomers of the invention may be administered to patients as a combination of two or more different anticode oligomer oligodeoxynucleotide sequences or as a single type of sequence. For instance,

5   TI-AS and SD-AS could be administered to a patient or TI-AS alone.

It is also believed that the anticode oligomers of the invention may be useful in the treatment of autoimmune diseases. Autoimmune diseases are those

10   diseases in which the body's immune system has malfunctioned in some way. Administration of the anticode oligomers of the invention to a person having an autoimmune disease should inhibit proliferation of bcl-2 overexpressing lymphocytes, which would in turn reduce

15   the symptoms of the autoimmune disease. For use in treating autoimmune diseases,' the anticode oligomers would be administered as described herein.

EXAMPLES

General Methods

20   The Examples below use the following protocols:
A. Cells and Cell Cultures. Human leukemic cells lines used for these studies were RS11846 follicular lymphoma cells, 697 pre-B cell acute lymphocytic leukemic cells, and JURAT T cell acute

25   lymphocytic leukemic cells as described in Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986) and Weiss et al., *Proc. Natl. Acad. Sci. USA*, 138:2169-2174 (1987). Human peripheral blood lymphocytes (PBL) were isolated from fresh whole blood as described in Reed et

30   al., *J. Immunol.*, 134:314-319 (1985). All lymphoid cells were cultured at 5×10^5 cells/ml in RPMI medium supplemented with 1 mM glutamine, antibiotics, and either 5-10% (v:v) fetal bovine serum (FBS), 5-10% (v:v) calf serum (CS) (both from Hyclone Laboratories), or 1% (v:v)

35   HLI concentrated supplement (Ventrex Laboratories) for

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serum-free cultures. Murine fibroblast cell lines were
added at 10^3 cells/cm^2 in DMEM medium containing
glutamine, antibiotics and 5-10% (v:v) FCS. Fibroblast
cell lines were NIH 3T3 cells, 3T3-B-alpha-S cells, and
3T3-B-alpha-AS cells. These latter two cell lines are
NIH 3T3 cells that express high levels of a human
bcl-2-alpha cDNA in either the sense or antisense
orientation, respectively, by virtue of stable
transfection with expression vectors constructs.

B.   Measurement of Cellular Growth. Growth of
cell lines cultured in the presence or absence of
anticode oligomers was measured by two methods: cell
counts using a hemocytometer; and DNA synthesis by
assaying [^{3}]-thymidine incorporation essentially as
described in Reed et al., *J. Immunol.*, 134:314-319
(1985). Briefly, cells were cultured in 96-well flat-
bottomed microtiter plates (Falcon) at 0.2 ml/well. At
appropriate times, cells were resuspended, 25 μl removed
from cultures for cell counting, and this volume replaced
with 25 μl of 20 UCi/ML [^{3}H]-thymidine (specific activity
6.7 Ci/mmole) (New England Nuclear). Microtiter cultures
were then returned to 37°C and 95% air: 5% CO_2 atmosphere
for 8 hours before lysing cells an glass filters and
determining relative levels of [^{3}H]-thymidine
incorporation into DNA by scintillation counting. Cell
counts were performed in the presence of trypan blue dye
to determine the concentration of viable cells in
duplicate microcultures.

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl
tetrazolium bromide)] dye reduction assays were performed
by the method of Tada, et al. J. Immunol Methods 93, 157
(1986), and confirmed to be within the linear range of
the assay under the conditions described here. The
number of viable cells per well was extrapolated from
standard curves that were included with each assay and

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that consisted of serial two-fold dilutions of
exponentially growing SU-DHL-4 cells in HL-1 medium,
beginning with 10^6 cells/ml (0.2mi/well). Samples were
assayed in triplicate and the OD600$_{nm}$ for a media/reagent
5   blank was subtracted from all values prior to
calculations.

        C.   RNA Blot Analysis. Total cellular RNA was
isolated by a quanidinium isothiocyanate/phenol procedure
as described in Chomczynski et al., *Analyt. Biochem.*,
10   162:156-139 (1987). The polyadenylated fraction was
purified by oligodeoxythymidine-cellulose chromatography
as described in Aviv et al., *Proc. Natl. Acad. Sci. USA*,
69:1408-1412 (1972). Approximately 5 μg aliquots of mRNA
were size-fractionated in 0.8% agarose/6% formaldehyde
15   gels and transferred to nylon membranes. Blots were
prehybridized, hybridized, and washed exactly as
described in Reed et al., *Mol. Cell Biol.*, 5:3361-3366
(1985), using either a ^{32}P-cDNA for human *bcl*-2, as
described in Tsujimoto et al., Proc. Natl. Acad. Sci.
20   USA, 83:5214-5218 (1986), or a murine *bcl*-2 probe,
pMBCL5.4 as described in Negrini et al., *Cell*, 49:455-463
(1987). Blots were exposed to Kodak XAR film with
intensifying screens at -70°C for 1-10 days. Eluting
^{32}P-*bcl*-2 probes from membranes and rehybridizing with a
25   ^{32}P probe for mouse beta-2-microglobulin verified nearly
equivalent amounts of mRNA for all samples on blots.

                        EXAMPLE 1
                Preparation of Anticode oligomers
                Normal and phosphorothioate
30   oligodeoxynucleotides were synthesized using an Applied
Biosystems 380B DNA synthesizer, and purified by HPLC
reverse-phase chromatography (PRP-1 column) as described
in Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988)
which is specifically incorporated as if fully set forth
35   herein. In some cases it was necessary to further purify

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oligodeoxynucleotides by C18-Sep-Pak chromatography
(Waters Associates, Millipore, Inc.), as described
previously in Kern et al. , *J. Clin. Invest.*, 81:237-244
(1988), to eliminate nonspecific cytotoxic activity.

5   Oligodeoxynucleotides eluted in 30% acetonitrile were
evaporated to dryness, resuspended at 1-2 mM in sterile
Dulbecco's phosphate-buffered saline or Hanks' buffered
salt solution (both from Gibco), and stored at -80°C in
small aliquots.

10   Table 1 shows the oligodeoxynucleotides
synthesized and their relation to the sense-strand of the
human *bcl*-2 gene. Portions of the sequence of the coding
strand of the human *bcl*-2 gene are shown, including the
translation initiation site (top), splice donor site

15   (middle), splice acceptor region (bottom), and
emperically selected sites within the 5' untranslated
portion of bcl-2 pre-mRNA. The ATG initiation codon, GT
splice donor, and AG splice acceptor consensus sequences
are in boxes.

20   The sequences of the oligodeoxynucleotides
synthesized for these investigations are presented, and
their relation to human *bcl*-2 mRNA is indicated. The
TI-AS oligodeoxynucleotide is antisense at the
translation initiation site and TI-S is its complementary

25   sense version. SD-AS and SD-S are oligodeoxynucleotides
having antisense and sense orientations, respectively,
relative to the splice donor region.

The oligodeoxynucleotide TI-AS straddles the
predicted translation-initiation site of *bcl*-2 mRNAs and

30   is complementary (antisense) to this region. As a
control, the sense version of this 20 bp
oligodeoxynucleotide, TI-S, was also synthesized.

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In an effort, to specifically block splicing of
bcl-2 mRNAs, a 20 bp antisense oligodeoxynucleotide,
SD-AS, was synthesized that overlaps the splice donor
site in *bcl*-2 primary transcripts. In addition, a
5   complementary sense oligodeoxynucleotide, SD-S, was
prepared as depicted in Table 1. The human *bcl*-2 gene
gives rise to several transcripts through alternative
splice site selections, see Tsujimoto et al., *Proc. Natl.
Acad. Sci. USA*, 83:5214-5218 (1986). The preponderance
10   of these transcripts depend upon splicing and encode a 26
kDa protein, *bcl*-2-alpha. One minor transcript, however,
does not undergo a splice and consequently encodes a 22
kDa protein *bcl*-2-beta. The SD-AS oligodeoxynucleotide
can thus potentially block maturation of most but not all
15   *bcl*-2 transcripts.

EXAMPLE 2
Treatment of Serum for In Vitro Investigations
of Antisense Normal Oligodeoxynucleotides
Because normal oligodeoxynucleotides are
20   sensitive to degradation by nucleases present in serum,
the efficacy of the TI-AS oligodeoxynucleotide in fetal
bovine serum (FBS) heated for 30 minutes at 56°C (the
usual procedure for inactivating serum complement) was
contrasted with the efficacy of TI-AS in FBS heated for
25   1 hour at 68°C, a temperature sufficient for irreversible
inactivation of many nucleases. The RS11846 follicular
lymphoma cell line was used. RS11846 cells contain a t
(14; 18) chromosomal translocation that deregulates *bcl*-2
expression, resulting in the accumulation of high levels
30   of *bcl*-2 mRNAs, Tsujimoto et al., *Proc. Natl. Acad. Sci.
USA*, 83:5214-5218 (1986).

RS11846 follicular lymphoma cells were cultured
in medium containing 5% (vol:vol) fetal bovine serum
(FBS) that had been heated at 56°C for 0.5 hours or at
35   68°C for 1 hour. TI-AS normal oligodeoxynucleotide was

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added at the initiation of cultur , and the density of
viable cells determined two days later.

          The TI-AS normal oligodeoxynucleotide was more
effective in 68 C-treated serum at suppressing the growth
in culture of these lymphoma cells. In all subsequent
experiments, sera heated at 68°C for 1 hour prior to use
were used in cultures. This treatment did not impair the
growth-supporting capacity of the sera.

                           EXAMPLE 3
              Specific Inhibition of Lymphoid Cell Growth
                 by Antisense Normal Oligodeoxynucleotides
          Antisense normal oligodeoxynucleotides directed
against the translation initiation site (TI-AS) and the
splice donor site (SD-AS) of bcl-2 transcripts were
tested for their ability to suppress the proliferation of
normal and neoplastic lymphoid cells.

          RS11846 follicular lymphoma cells, JUKRAT
T cell leukemia cells, and freshly isolated peripheral
blood lymphocytes were cultured in medium containing 10%
(vol:vol) FBS that had been heated at 68°C for one hour.
various concentrations of normal oligodeoxynucleotides
were added at the initiatIon of culture, including:
TI-AS, TI-S, SD-AS, and SD-S. Relative DNA synthesis was
measured in cultures after 2-3 days by [^{3}H]-thymidine
incorporation. Data were calculated as a percentage of
control cultures containing volumes of PBS or HBSS
equivalent to oligodeoxynucleotide-treated cultures, and
represent the mean (± standard deviation) of duplicate
cultures.

          Similar data were obtained by measuring cell
counts, excluding cold thymidine inhibition as an
explanation for the suppression of DNA synthesis observed
in cultures treated with antisense oligodeoxynucleotides.

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As shown in Figures 1(a), (b), (c) and (d) both the TI-AS and SD-AS óligodeoxynucleotides inhibited the growth of RS11846 cells in a concentration-dependent manner. The SD-AS oligonucleotide was less effective in

5   inhibiting cell growth than the TI-AS oligodeoxynucleotide. In contrast to these antisense oligodeoxynucleotides, sense oligodeoxynucleotides (TI-S and SD-S) were not inhibitory even at concentrations of up to 250 μG/ml. Moreover, non-sense

10   oligodeoxynucleotides (i.e., those having the same base composition as the antisense oligodeoxynucleotides but with scrambled sequences) also failed to suppress the proliferation of RS11846 cells. The data thus indicate that antisense oligodeoxynucleotides can specifically

15   block the proliferation of these tumor cells. Several other leukemic cell lines that express the *bcl*-2 gene were also tested for inhibition of their proliferation by TI-AS and SD-AS oligonucleotides. As with the JURKAT T cell acute lymphocytic leukemic cells, in every case a

20   specific and concentration-dependent decrease in the growth of these human leukemic cells in cultures containing antisense oligodeoxynucleotides was observed.

It has been demonstrated that *bcl*-2 expression is transiently induced in normal human peripheral blood

25   lymphocytes (PBL) when these cells are stimulated to proliferate, suggesting that this gene may play a role in the regulation of normal lymphocyte growth, Reed et al., *Science* 236:1295-1297 (1987). The capacity of antisense oligodeoxynucleotides to impair the growth of PBL

30   cultured with a monoclonal antibody, OKT3 (Van den Elsen et al., *Nature* 312:413-418 (1984)), that stimulates their proliferation was therefore tested. PBL were stimulated with 50 μl of purified OKT3 monoclonal antibody. As shown in Figures 1 (a), (b), (c) and (d) the TI-AS

35   oligodeoxynucleotide specifically suppressed the proliferation of PBL in a concentration-dependent manner.

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These antisense normal oligodeoxynucleotides thus
suppressed the growth in culture of leukemic cells that
constitutively express the *bcl*-2 gene and of normal
lymphocytes where in *bcl*-2 expression is inducible.

5　　　　　　　　　　　　　　　EXAMPLE 4
Time-Course of Inhibition by
Antisense Normal Oligodeoxynucleotides
The kinetics of inhibition by antisense
oligodeoxynucleotides was examined in cultures of RS11846
10　　follicular lymphoma cells and of 697 pre-B cell acute
lymphocytic leukemia cells. Both of these neoplastic
B cell lines transcribe and accumulate *bcl*-2 mRNAs at
high levels, Tsujimoto et al., *Proc. Natl. Acad. Sci.
USA*, 83:5214-5218 (1986).

15　　　　　　RS11846 follicular lymphoma and 697 pre-B cell
leukemia cells were cultured in medium containing 10%
(vol:vol) 68°C-treated FBS and normal
oligodeoxynucleotides. Cells were cultured with 50 μg/ml
TI-AS, 100 μg/ml SD-AS, 50 μg/ml TI-S (RS11846 cells) or
20　　100 μg/ml SO-S (697 cells), or PBS as a control. DNA
synthesis (kcpm/10^5 viable cells) and cell densities (10^5
viable cells/ml) were measured at various times after
initiation of cultures.

　　　　　　Antisense normal oligodeoxynucleotides markedly
25　　inhibited DNA synthesis measured in cultures of these
cells within 24 hours. Diminished cell densities were
readily apparent in these cultures within 2 days.
Antisense normal oligodeoxynucleotides thus rapidly
inhibited the *in vitro* growth of leukemic cells. The
30　　action of antisense oligodeoxynucleotides was specific,
since sense oligodeoxynucleotides did not impair
proliferation in these cultures. Though cell viabilities
often declined during the later days of culture no
increase in cell death was seen during the first 1-2 days

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of culture with antis nse oligodeoxynucleotides,
suggesting a non-cytotoxic mechanism.

EXAMPLE 5

Comparision of Different Serum Preparations

5   Inhibition of proliferation of leukemic cells
with antisense oligodeoxynucleotides can vary greatly
depending on the lot of serum used in cultures.

        To determine the effects of serum of inhibition
of proliferation, relative levels of DNA synthesis were
10   measured in cultures of 697 pre-B cell leukemia cells
2 days after addition of 200 μM TI-AS normal
oligodeoxynucleotide. Cells were cultured in medium
supplemented with 1% (vol:vol) HL1-concentrate
(serum-free condition), 5% (vol:vol) of two different
15   lots of calf serum (CS1 and CS2), or 5% (vol:vol) of two
different lots of fetal bovine serum (FBS1 and FBS2).
All sera were heated at 68°C for 1 hour prior to use in
cultures.

        The normal TI-AS oligodeoxynucleotide markedly
20   inhibited DNA synthesis (92%) and cellular proliferation
in serum-free cultures (HL1) of 697 cells. This
antisense oligodeoxynucleotide was equally effective
(94%) in cultures containing 5% (v:v) of one of the lots
of fetal bovine serum (FBS2). In contrast, inhibition
25   was significantly reduced in cultures containing other
serum preparations (CS1, CS2, FBS1). It has been
generally observed that antisense normal
oligodeoxynucleotides are less effective in cultures
supplemented with calf serum (CS) than in those
30   .containing fetal bovine serum (FBS).

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EXAMPLE 6

Concentration Dependence of Inhibition by Antisense
Normal Oligodeoxynucleotides in Serum-Free Cultures

697 pre-B cell leukemia cells were cultured in
medium with either 1% (vol:vol) HL1-concentrate
(serum-free conditions or 5% (vol:vol) 68°C-treated
FBS2). Relative levels of DNA synthesis and cellular
densities measured after 2 days in cultures containing
various concentrations of normal TI-AS
oligodeoxynucleotide.

The TI-AS oligodeoxynucleotide was inhibitory
at lower concentrations when used in serum-free cultures.
At 100 μM, for instance, no inhibition of cellular
proliferation was seen in FBS2-containing cultures,
whereas cell counts were reduced by approximately 75% in
serum-free cultures. At higher concentrations of
antisense oligodeoxynucleotides (200-250 μM), however,
inhibition of 697 cellular proliferation was comparable
in both types of cultures. The increased efficacy of
normal oligodeoxynucleotides in serum-free cultures was
specific, since the sense oligonucleotide (TI-S) was not
inhibitory at the same concentrations.

EXAMPLE 7

Antisense Phosphorothioate Oligodeoxynucleotides:
Time Course of Inhibition

To contrast the efficacy of phosphorothioate
oligodeoxynucleotides with that of normal
oligodeoxynucleotides with regard to inhibition of human
leukemic cell growth, phosphorothioate
oligodeoxynucleotides were cultured with 697 pre-B cell
leukemia cells and the effects on inhibition were
measured. 697 pre-B cell leukemia cells were cultured in
serum-free medium for various times before measuring DNA
synthesis (kcpm) and cell densities (10^6 cells/ml). Cells
were seeded at an initial density cf either 0.2×10^5

cells/ml or 0.5x10^5 cells/ml. Culture conditions were
25 μM TI-AS phosphorathioat , 25 μM TI-S
phosphorothioate, and control cultures treated with HBSS.

5   To avoid experimental variation due to
differences among lots of sera, 697 leukemic cells were
cultured in serum-free conditions. When cultured at an
initial seeding density of 0.5x10^6 cells/ml, 697 cells
achieved maximal DNA synthesis and cellular densities at
4-5 days. Addition of 25 μM sense phosphorothioate
10   oligodeoxynucleotide (TI-S) at the initiation of these
cultures had little effect on 697 cell growth. In
replicate cultures containing 25 μM antisense
phosphorothioate (TI-AS), however, some diminution in DNA
synthesis was evident within 2 days and was maximal at
15   4-5 days. Maximal inhibition of 697 cell growth, as
determined by cell counts, was seen at 6 days after
initiation of cultures.

When 697 cells were initially seeded at 0.2x10^6
cells/ml, the antisense phosphorothioate
20   oligodeoxynucleotide, TI-AS, resulted in only slight
inhibition at 2 days, attaining maximal suppression of
DNA synthesis in these cultures at day 7. As with normal
oligodeoxynucleotides, this inhibition by
phosphorothioate oligodeoxynucleotides appeared to be
25   mediated through non-cytotoxic mechanisms, since cellular
viabilities did not decline until late in the course of
culture. Compared with normal antisense
oligodeoxynucleotides, therefore, phosphorothioate
oligodeoxynucleotides had a slower onset of action.

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EXAMPLE 8

Concentration Dependence of Inhibition by Antisense bcl-2 Phosphorothioate Oligodeoxynucleotides

The concentration descendence of inhibition by
5   phosphorothioate and normal TI-AS oligodeoxynucleotides
in cultures of 697 cells in serum-free medium was
compared as follows.

697 cells were cultured in serum-free medium
for either 3 days (normal oligodeoxynucleotides) or
10   4 days (phosphorothioate oligodeoxynucleotides) prior to
measuring cell densities and levels of DNA synthesis.
Oligodeoxynucleotide additions to cultures included TI-AS
phosphorothioate, TI-S phosphorothioate, TI-AS normal,
and TI-S normal.

15   As shown in Figures 2 (a) and (b), TI-AS
phosphorothioate oligodeoxynucleotides markedly inhibited
the proliferation of 697 cells at 25-50 μM. In contrast,
normal TI-AS oligodeoxynucleotides required
concentrations 5- to 10-fold higher (approximately 250
20   μM) to cause a comparable suppression of 697 cellular
proliferation. Suppression by the antisense
phosphorothioate oligodeoxynucleotide TI-AS was specific
over this concentration range, since its complementary
sense oligodeoxynucleotide (TI-S) produced little
25   inhibition of 697 cell growth in replicate cultures (see
Figures 2(a) and (b).

EXAMPLE 9

Influence of Serum Preparation on Inhibition by Antisense Phosphorothioate Oligodeoxynucleotides

30   To further define the effects of serum
preparation on the inhibitory activity of
phosphorothioate oligodeoxynucleotides, FBS that had been
heated to 56°C for 30 minutes, 68°C for 1 hour, or not

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heated prior to addition to cultures was added to
cultures of RS11846 lymphoma cells.

    RS11846 cells were cultured in medium
containing 1% (vol:vol) HL1-concentrate or 5% (vol:vol)
5   FBS that had been heated at 56°C for 0.5 hour, 68°C for
1 hour, or that had not been heated. Cell counts were
calculated as a percentage relative to control cultures
treated with equivalent concentrations of TI-S
phosphorothioate oligodeoxynucleotide, and represent the
10   mean percentage (standard deviation was less than 10% for
all values) for duplicate cultures counted on days 4
and 5.
    The TI-AS phosphorothioate oligodeoxynucleotide
completely inhibited the growth of RS11846 cells at 25
15   μM, with an estimated half-maximal inhibitory
concentration of approximately 11 μM. In contrast, this
phosphorothioate oligodeoxynucleotide was considerably
less effective in cultures containing 5% (v:v) FBS.
Furthermore, heating FBS prior to adding it to cultures
20   did not significantly improve the ability of the TI-AS
phosphorothioate oligodeoxynucleotide to suppress the
growth of RS11846 lymphoma cells. At an
oligodeoxynucleotide concentration of 50 μM, inhibition
of proliferation of RS11846 cells never exceeded 48%
25   serum-containing cultures, regardless of the heating
procedure used.

EXAMPLE 10
Influence of Dialysis of Serum on Inhibition by Normal
and Phosphorothioate Antisense Oligodeoxynucleotides
30   To further characterize the nature of the
interfering substances in serum, experiments were
performed wherein 68°C-heated serum was extensively
dialyzed (molecular weight cutoff = 3500) prior to being
added to cultures of 697 leukemic cells. Experiments
35   were conducted with 12.5 μM TI-AS phosphorothioate

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oligode xynucleotide and 200 μM of the normal
oxygen-based TI-AS oligcdeoxynucleotide.

5

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697 cells were cultured in medium containing 1%
(vol:vol) HL1-concentrate (A) or 5% (vol:vol) of three
different lots of 68°C-treated FBS (B,C,D). Each serum
preparation was contrasted before (ND) and after (D)
extensive dialysis. TI-AS (+) and TI-S (-)
oligodeoxynucleotides were added to replicate cultures at
200 μM for normal oxygen-based oligodeoxynucleotides
(OXY) and at 12.5 uM for phosphorothioate
oligodeoxynucleotides (PT). Relative levels of DNA
synthesis (kcpm) were measured after 2 or 4 days of
culture for normal and phosphorothioate
oligodeoxynucleotides, respectively.

15

20

For the three different lots of FBS tested, two
exhibited little change after dialysis in cultures
containing either normal or phosphorothioate
oligodeoxynucleotides. One lot of FBS, however, appeared
to interfere less with the inhibitory activities of these
antisense oligodeoxynucleotides after dialysis.

EXAMPLE 11
Experiments with Stably Transfected NIH 3T3 Cells

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Though the antisense oligodeoxynucleotides
described herein were designed to block *bcl-2* mRNA
translation (TI-AS) and splicing (SD-AS), the molecular
mechanisms of their actions are not yet known. To
determine the effect of formation of
oligodeoxynucleotide-RNA hybrids within cells upon
inhibition of cellular growth, irrespective of the
nucleotide sequence, cells transformed to express human
bcl-2 cDNA transcripts were cultured with normal
oligodeoxynucleotides.

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200 μM of normal TI-AS and TI-S
oligodeoxynucleotides were added to cultures of typical
NIH 3T3 cells and to cultures of th se cells that had
been stably transfected with expression constructs that
5   produce high levels of human *bcl*-2 cDNA transcripts for
either the usual sense (3T3-alpha-S cells) or the
antisense (3T3-alpha-AS cells) strand.

For RNA blot analyses, polyadenylated mRNA was
purified from normal NIH 3T3 cells and from cells stably
10   transfected with expression constructs that produce
either sense (3T3-alpha-S) or antisense (3T3-alpha-AS)
recombinant *bcl*-2-alpha mRNAs, according to the method of
13. Approximately 5 μg of mRNA was subjected to RNA blot
analysis, essentially as described in (16), using either
15      $^{3}_{2P}$-labeled hybridization probes derived from human or
murine *bcl*-2 sequences.

An autoradiogram resulting from a one-day
exposure of a blot containing RNAs from normal 3T3 cells,
3T3-alpha-AS cells, and 3T3-alpha-S cells showed high
20   relative levels of recombinant 2.4 and 1.4 kbp *bcl*-2
transcripts produced from the *bcl*-2 expression constructs
that were transfected into 3T3-alpha-AS and 3T3-alpha-S
cells.

A 10-day exposure of a blot containing RNA from
25   normal 3T3 cells that were either proliferating or
quiescent at the time of harvesting RNA showed low but
detectable levels of normal 7.5 and 2.4 kbp murine *bcl*-2
transcripts present in proliferating 3T3 cells.

TI-AS oligodeoxynucleotide specifically
30   suppressed DNA synthesis and cellular replication in
cultures of normal NIH 3T3 cells, c nsistent with
findings by others that fibroblasts do contain *bcl*-2
transcripts, albeit at low levels. The TI-AS

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oligodeoxynucleotide disclosed herein is complementary to
the mouse *bcl*-2 sequence in 18 of its 20 bases (17),
accounting for its ability to suppress the growth of
murine NIH 3T3 cells.

5   NIH 3T3 cells, 3T3-alpha-AS cells, and
3T3-alpha-S cells were cultured in medium containing 5%
(vol:vol) 68°C-treated serum and either HBSS, 200 µM TI-S
normal oligodeoxynucleotide, or 200 µM TI-AS normal
oligodeoxynucleotide. Relative levels of DNA synthesis
10   (kcpm) were measured in cultures after 3 days and reflect
a 16 hour incubation with 0.5 µci/well of [^{3}H]-thymidine.
Cell densities, estimated by phase microscopy, were
consistent with the measured DNA synthesis in cultures.
The percentage of inhibition of DNA synthesis in cultures
15   containing TI-AS oligodeoxynucleotides was calculated
relative to control cultures containing HBSS.

          As with normal NIH 3T3 cells, culturing
3T3-alpha-S cells (producing human *bcl*-2-alpha sense
transcripts) with TI-AS and TI-S oligodeoxynucleotides
20   demonstrated specific suppression, since the sense
oligodeoxynucleotide TI-S was not inhibitory. The level
of inhibition of cellular proliferation by the antisense
oligodeoxynucleotide, however, was not as great in
3T3-alpha-S cells, as might be expected, since these
25   cells contain more *bcl*-2 mRNA.

          Adding TI-S oligodeoxynucleotide to cultures of
3T3-alpha-AS cells (produce antisense *bcl*-2 transcripts)
ruled out inhibition of cellular growth through a
nonspecific mechanism involving oligodeoxynucleotide--RNA
30   hybrid formation. The TI-S oligodeoxynucleotide caused
little suppression of 3T3-alpha-AS cell proliferation,
whereas the TI-AS oligodeoxynucleotide was markedly
inhibitory in these cells. Similar data were obtained

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with TI-AS and TI-S phosphorothioate
oligodeoxynucleotides.'   •   •

EXAMPLE 12
Measurements of DNA Fragmentation as an Indicator of
bcl-2 Antisense Oligodeoxynucleotide-Mediated Programmed
Cell Death in Human Lymphoma Cels

Oligonucleotides having the sequences shown in
Table 2 were tested for the ability to induce programmed
cell death (DNA fragmentation) in the human
t(14:18)-containing human lymphoma cell line RS11846.
The oligonucleotides were all phosphodiesters, and were
targeted against the translation initiation site or the
5'-cap region of *bcl*-2 pre-mRNAs. Control
oligodeoxynucleotides included a *bcl*-2 sense version
(TI-S) of Tl-AS (having SEQ ID NO: 7) and a scrambled
version of TI-AS that has the same base composition, but
with jumbled nucleotide order.

TABLE 2

SEQUENCE		SEQ ID NO:
CGCGTGCGAC	CCTCTTG	8
TACCGCGTGC	GACCCTC	9
CCTTCCTACC	GCGTGCG	11
GACCCTTCCT	ACCGCGT	12
GGAGACCCTT	CCTACCG	13
GCGGCGGCAG	CGCGG	14
CGGCGGGGCG	ACGGA	15
CGGGAGCGCG	GCGGGC	16

RS11846 cells were adapted to grow in HL1 media
with 1% FCS and their DNA metabolically labeled by
addition of ^{125}I-deoxyuridine to cultures for three hours.
Labeled cells were then washed thoroughly and cultured
for two days in the presence of various oligonucleotides
at 50 AM. Cells were then recovered from 200 μL cultures
by centrifugation, and lysed in a hypotonic buffer

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containing 10 mM EDTA and 14 Triton X100. After
centrifugation at 16,000 xg to pellet unfragmented
genomic DNA, the supernatant fraction containing
fragmented DNA was extracted with phenol/chloroform and
5   ethanol precipitated. This DNA was then subjected to gel
electrophoresis in 1.5% agarose gel and transferred to
nylon membranes for autoradiography.

          The results of two experiments are shown in
     Figures 3 and 4(a-b). The six *bcl*-2 antisense
10   oligonucleotides targeted in the vicinity of the ATG site
     of translation initiation in *bcl*-2 mRNAs were tested.
     "C-Oligo-2" refers to an oligonucleotide with 4
     purposeful mismatches. "U" indicates untreated control
     cells. Figures 4 (a-b) show the results for the
15   oligonucleotides shown in Figure 3. "Sc20" refers to a 20
     mer with the same base composition as TI-AS, but with
     scrambled sequence. Figure 4(b) shows the results for
     three oligonucleotides
     targeted against the 5'-cap of *bcl*-2 mRNAs. The numbers
20   refer to the distance of these oligomers from the
     ATG-translation initiation site.

          The presence of a ladder of DNA fragments (unit
     size of approximately 200 bp) is indicative of programmed
     cell death. At 50 μM, TI-AS caused little DNA
25   fragmentation, whereas the oligonuoleotides having SEQ ID
     NO: 9 and SEQ ID NO: 10, and one of the 5'-cap
     oligonucleotides (SEQ ID NO: 14) led to pronounced DNA
     fragmentation.

                         EXAMPLE 13
30   Concentration-Dependence of Inhibition by Antisense
            Phosphodiester Oligodeoxynucleotides in Serum-Free
     Cultures
               697 pre-B cell leukemia cells were cultured in
     medium with either 1% (vol:vol) HL-1 concentrate

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(serum-free conditions [o] or 3% (vol:vol) 68°C-treated
serum (FBS2) [___], see Figure 5. Shown are cellular
densities measured after 2 days in cultures containing
various concentrations of phosphodiester TI-AS
5   oligodeoxynucleotide. Data are shown as percentages
relative to control cultures treated with a sense
oligonucleotide, and reflect the mean ± standard
deviation for duplicate samples.

EXAMPLE 14

10   ## Immunofluorescence Analysis of bcl-2 Protein Levels in
Oligodeoxynucleotide-Treated 697 Cells

For studies with oligodeoxynucleotides, 0.25×10^4
(for phosphorothioate) or 0.5×10^5 (for normal
oligodeoxynucleotides), 697 cells were cultured in 1 ml
15   of HL-1 serum-free medium in 24 well culture dishes
(Linbro. Flow Lab, Inc.). After 2 days (for normal) or
4 days (for phosphorothioates), cells were recovered from
cultures, washed once in [PBS, pH 7.4 (Gibco) - 0.1%
bovine serum albumin - 0.1% sodium azide], and fixed for
20   5-10 minutes on ice in 1% paraformaldehyde/PBS solution.
The cells were then washed once in PBS and incubated in
1 ml of absolute methanol at 20°C for 10 minutes. After
washing once in PBS-A, cells were then resuspended in PBS
containing 0.05% Triton-X100 for 3 minutes on ice, washed
25   in PBS-A and preblocked for 30 minutes at 4°C in PBS with
10% (v/v) heat-inactivated goat serum.

For addition of the first antibody, preblocked
cells were resuspended in 100 μl of PBS-G (PBS-1% goat
serum-0.1% sodium azide) prior to aliquoting 50 μl into
30   separate tubes that contained 1 μl of either BCL2
antibody (Halder et al., *Nature* (London), 342:195-197
(1989)) or affinity-purified normal rabbit control IgG
(Cappel 6012-0080) and incubated for 1 hour on ice. The
BCL2 antibody used for these studies was prepared in
35   rabbits using a synthetic peptide corresponding to amino

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acids (98-114) of the BCL2 protein and was affinity--
purified by protein-A-Sepharose chromatography and used
at approximately 1 mg/ml. Cells were then washed in
PBS-A and incubated in 0.5-1.0 ml PBS-A for 15-20 minutes
5   on ice to allow diffusion of nonspecific cell-associated
antibody prior to resuspending cells in 100 μl of PBS-G
containing 5 μg of biotinylated scat anti-rabbit IgG
(BA1OOO; Vector Labs) for 30 minutes. After washing once
and incubating for 15 minutes in PBS-A, cells were
10   finally resuspended in 100 μl of PBS-A containing 2 μg of
FITC-conjugated avidin (Vector Labs A2011) for 20 minutes
and washed three times in PBS-A prior to analysis with an
Ortho cytofluorograph 50-H connected to an Ortho 2150
data-handling system. The specificity of method for
15   detecting BCL2 protein was confirmed by
immunofluorescence microscopy (showing cytosolic stain
peptide competition, and studies of cell lines that
expressed various levels of BCL2 mRNA and proteins
through gene transfer manipulations.

20   For measurements of surface HLA-DR antigen
expression, an indirect immunofluorescence assay method
was used (Reed et al., *J. Immunol.* 134:1631-1639 (1985))
involving incubation of viable cells with a murine
anti-HLA-DR monoclonal antibody (IgG2a) (Becton-Dickinson
25   7360) or a negative control antibody, R3-367 (IgG2a),
followed by FITC-conjugated scat anti-mouse IgG (Cappel
1711-0081). Cells were fixed in 1% paraformaldehyde/PBS
prior to FACS analysis.

               697 cells were cultured for 2 days (PO) or
30   4 days (PS) with various oligonucleotides. In
Figures 6(a-b), the black columns show the results with a
sense oligonucleotide, and the hatched columns with an
antisense oligonucleotide TI-AS. Cells were labeled with
anti-*bcl*-2 antiserum and analyzed by FACS. Data are

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expressed as percentages relative to the mean
fluorescence obtained with untreated 697 cells.

5   Figures 7(a-d) show typical FACS results
obtained for 697 cells before and after treatment with
100 μM PO *bcl*-2 antisense oligonucleotides. A:
untreated 697 cells labeled with either anti-*bcl*-2
antiserum (hatched area) or normal rabbit serum control
(white area); B: untreated 697 cells labeled with either
anti-HLA-DR antibody (hatched area) or a negative control
10   antibody (white area); C: 697 cells cultured for 2 days
with either normal *bcl*-2 TI-AS (white area) or TI-AS
(hatched area) oligodeoxynucleotides and labeled with
anti-*bcl*-2 antibody; D: 697 cells cultured with TI-AS
and TI-S oligodeoxynucleotides (as in C), but labeled
15   with anti-HLA-DR antibody.

As shown in Figures 6 (a) and (b) , PO and PS
bcl-2 antisense oligonucleotides produced specific
concentration-dependent reductions in the levels of *bcl*-2
proteins, without altering the levels of expression of
20   HLA-DR (Figures 7(a-d)) and other control antigens. At
150 μM, for example, PO antisense oligodeoxynucleotide
caused an approximately 75-95% reduction in *bcl*-2
fluorescence, whereas the control sense
oligodeoxynucleotide diminished *bcl*-2 protein levels by
25   only 10-20% (Figure 6(a)). Similarly, cultured 697 cells
for 4 days with the PS antisense oligodeoxynucleotide ar
25 μM resulted in approximately 70% reduction in *bcl*-2
fluorescence. In comparison, the sense PS
oligodeoxynucleotide TI-AS inhibited *bcl*-2 protein levels
30   by only approximately 15%, as measured by this assay
(Figure 6(b)).

SIGNIFICANCE
        In phosphorothioate oligodeoxynucleotides, one
of the non-bridging oxygen atoms in each internucleotide

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phosphate linkage is resolaced by a sulfur atom. This
m dification renders phosphorothioate
oligodeoxynucleotides extremely resistant to cl avage by
nucleases, Stein et al., *Nucl. Acids Res.*, 16:3209-3221

5   (1988). Despite the substitution of a sulfur atom for an
oxygen, phosphorothioate oligodeoxynucleotides retain
good solubility in aqueous solutions; hybridize well,
though with some decrease in the melting temperature of
RNA-oligodeoxynucleotides duplexes; and are synthesized

10   conveniently by the widely employed method of automated
oligodeoxynucleotides synthesis with phosphoroamidites.

Antisense *bcl*-2 phosphorothioate
oligodeoxynucleotides have been found to be more potent
inhibitors of leukemic cell grown than their normal

15   oxygen-based counterparts. When tested under serum-free
conditions, these oligodeoxynucleotides reduced cellular
proliferation by half at concentrations of approximately
15-23 μM, whereas the normal oligodeoxynucleotide
achieved 50% inhibition at 125-250 μM. This finding may

20   be explained by the reduced sensitivity of
phosphorothioate oligodeoxynucleotides to cellular
nucleases, or may be attributable to other mechanisms.
For example, mRNAs hybridized with phosphorothioate
oligodeoxynucleotides may experience enhanced degradation

25   through a mechanism involving an RNAse H-like activity.

Despite their increased inhibitory activity,
phosphorathioate antisense oligodeoxynucleotides retained
sequence-specificity. At the concentrations tested (less
than 25 μM), sense versions of these

30   oligodeoxynucleotides had little effect on leukemic cell
growth. Both normal and phosphorothioate antisense
oligodeoxynucleotides appeared to initially suppress the
proliferation of leukemic cells through non-cytotoxic
mechanisms. During the first few days of culture,

35   cellular replication was inhibited without a concomitant

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rise in cell death.   Later in these cultures (days 4-5 for normal oligodeoxynucleotides, days 6-8 for phosphorothioates), however, cellular viabilities declined.

5   Comparing the kinetics of inhibition by normal and phosphorothioate oligodeoxynucleotides revealed that the latter compounds have a slower onset of action. Maximal inhibition of leukemic cell proliferation by normal antisense oligodeoxynucleotides occurred two days
10   after initiation of cultures, whereas phosphorothioate oligodeoxynucleotides required 4 to 7 days to achieve maximal inhibition.

        The usefulness of anticode oligomers in inhibiting human lymphoma/leukemia cells and other types
15   of cancer cells that express the *bcl*-2 gene has been shown by the examples herein. Anti-sense oligodeoxynucleotides complementary to at least an effective portion of the mRNA of the human *bcl*-2 gene has been found to inhibit growth of RS11846 human follicular
20   lymphoma cells t (14;18) chromosomal translocation and high *bcl*-2 expression), 697 human pre B cell leukemia cells (high *bcl*-2 expression), JURKAT human acute lymphocytic leukemia cells (medium *bcl*-2 expression), normal human lymphocytes (medium *bcl*-2 expression) and
25   murine fibroblasts (low *bcl*-2 expression). Although *bcl*-2 antisense reagents can suppress the growth of many types of cells, the t(14:18) lymphoma and leukemia cells seem to be the sensitive, allowing for specific inhibition of malignant cells.

30   As demonstrated in the following Examples, a variety of DNA analogs can be employed in the instant invention. For example, phosphorothioates, methylphosphonates, and mixed oligomers containing combinations of phosphodiesters and phosphorothioate or

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methylphosphonate nucleosides. It shoud be underst od
that RNA analogs can also be employed in the invention.

EXAMPLE 15
Methylphosphonate (MP)/Phosphodiester (PO) bcl-2
5   Antisense Oligomers Induce Death of DoHH2 Lymphoma Cells
           The purpose of this study was to determine the
efficacy of various analogs of the anticode oligomers for
inhibiting lymphoma cell survival.

           DoHH2 is a human lymphoma cell line containing
10   a t(14:18)-translocation that activates the bcl-2 gene.
DoHH2 cells were cultured for 3 days without oligomers or
in the presence of various concentrations of antisense
(As) and scrambled (Sc) methylphophonate
(MP)/Phosphodiester (PO) oligomers for 3 days. Cell
15   viablity was assessed by trypan blue dye exclusions, and
the data expressed as a percentage relative to DoHH2
cells cultured without oligomers. The MP/PO oligomers
was an 18-mer targeted against the first 6 codons of the
bcl-2 open reading frame in which 5 internal linkages
20   were phosphodiester and the flanking nucleosides were
methylphophonates.

           The results indicate that these anticode
oligomer analogs are potent and specific inhibitors of
lymphoma cell survival.

25   EXAMPLE 16
       Methylphosphonate (MP)/Phosphodiester (PO) Chimeric
                  Oligomers Inhibit Growth of MCF-7
                     Human Breast Cancer Cells
           The purpose of this study was to determine the
30   efficacy of the claimed anticode oligomer analogs to
inhibit the survival of solid tumor cells which highly
express bcl-2.

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MCF-7 is a human breast adenocarcinoma cell line
that c ntains relatively high levels of bcl-2 protein.
The cells were cultured at 4,000 cells per well in 96-
well microtiter plates in the presence or absence of
MP/PO oligomers. Relative cell numbers per well were
then estimated by MTT assay, based on a standard curve
prepared using freshly plated, untreated MCF-7 cells.
The antisense (As) and scrambled (Sc) MP/PO oligomers wer
the same as those described in Example 15. Data
represent the mean +/- standard deviation for
determinations.

The results demonstrate sequence specific
inhibition of growth of solid tumor cells by the the
claimed anticode oligomer analogs.

EXAMPLE 17
Optimimization of Anticode bcl-2 Oligomer Sequences
The purpose of this study was to determine
optimum target sites or sequence portions on mRNA for
inhibiting cell survival by contacting the cells with
various claimed anticode molecules whose sequences were
computer generated.

DoHH2 lymphoma cells were treated with various
concentrations of oligomers targeted to different sites
on the bcl-2 mRNA. The ATG oligomer targets the
translation initiation site, and is complementary to the
first 6 codons of the open reading frame. The Dscore 23
and Dscore 72 oligomers target sites in the 5'
untranslated region of the mRNA. Sc oligomers represent
negative controls having the same length and base
composition but in scrambled order. All oligomers were
prepared as phosphodiester (PO)/phosphorothioate (PS)
chimeras, where only the last (3') two internucleoside
linkages were phosphorothioates. Oligomers were added
directly to cultures and relative numbers of viable cells

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were estimated by MTT assay 3 days later. Data represent
mean +/- standard deviation.

        The results indicate that the Dscore 23
oligomer, targeted to the 5' untranslated region, has,
5   compared to the other anticode oligomers tested in this
Example, superior activity for inhibiting cell survival.

                        EXAMPLE 18
        Reveral of Chemoresistance of Tumor Cells by Antisense-
                Mediated Reduction of bcl-2 Gene Expression
10   The following work was undertaken to determine
if anticode oligomers directed against the expression of
the bcl-2 gene would reverse chemoresistance, that is to
say, increase the sensitivity to cancer chemotherapeutic
agents in cancer tumor cells expressing the bcl-2 gene.

15   High levels of bcl-2 protein appeared to
increase the relative resistance of lymphoid cells to
killing induced by a wide variety of cancer
chemotherapeutic agents including, but not limited to,
Ara-C, MTX, vincristine, taxol, cisplatin, adriamycin,
20   etoposide, mitozantron, 2-chlorodeoxyadenosine,
dexamethasone (DEX), and alkylating agents. (Miyashita,
T. and Reed, J.C., Cancer Res. 52:5407, October 1, 1992).
While these drugs have diverse biochemical mechanisms of
action, it is believed that all have in common the
25   ability to ultimately trigger cancer cell death by
activating endogenous cellular pathways leading to
apoptosis (Eastman, A. Cancer Cells 2:275 (1990)). It is
understood that the claimed anticode molecules and
analogs thereof as used herein are effective for their
30   intended purposes of enhancing sensitivity to cancer
chemotherapeutic drugs including, but not limited to,
antimetabolites, alkylating agents, plant alkaloids, and
antibiotics.

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Antimetabolites include, but are not limited to, methotrexate, 5-fluoruracil, 6-mercaptopurine, cytosine arabinoside, hydroxyurea, 20chlorodeoxy adenosine.

5   Alkylating agents include, but are not limited to, cyclophospham;ide, melphalan, busulfan, cisplatin, paraplatin, chlorambucil, and nitrogen mustards.

Plant alkaloids include, but are not limited to, vincristine, vinblastine, VP-16.

10   Antibiotics include, but are not limited to, doxorubicin (adriamycin), daunorubicin, mitomycin c, bleomycin.

Other cancer chemotherapeutic agents include DTIC (decarbazine), mAMSA, hexamethyl melamine, 15   mitroxantrone, taxol, etoposide, dexamethasone.

In the present work, both nuclease resistance phosphorothioates (PS) and phosphodiesters in which only the 3'-most internucleoside bond was a thioate linkage (PO/PS) were employed. The PO/PS oligomers are 20   resistance to 3' exonucleases (the principal nuclease activity of serum) and generally form more stable heteroduplexes with target RNAs.

Cationic lipids were used to improve the uptake and subsequent release of oligomers into effective 25   intracellular compartments, and are exemplary pharmaceutical carriers for the claimed anticode oligomers.

The methods for preparing and purifiying the antisense (AS) and scrambled (SC) 18'mer oligonucleotides 30   used for the present work are described above in General

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Methods and in Kitada et al. (<u>Antisense R & D</u>, 3:157
(1993)). Phosphodiester oligonucleotid s were
synthesized in a 10-15 micromole scale using
phosphoroamidate chemistry with oxidation by iodine, and
5   then purified using a C_{18}-reverse phase column. In most
cases, oligomers were additionally ethanol-precipitated
five times to eliminate any nonspecific cytotoxic
activity, and then dried and resuspended in sterile HL-1
medium (Ventrex Labs, Inc; Burlingame, CA) at 1-10 mM.
10   The pH of this solution was adjusted using 1-10 M NaOH
until the phenol red indicator dye in the media returned
to its original color.

The principal oligomers used were 18-mers,
having either the sequence:
15   I.   TCTCCCAGCGTGCGCCAT (SEQ ID NO. 17), which is
antisense to the first six codons of the human bcl-2
open reading frame (SEQ ID NO. 19); or

II.   TGCACTCACGCTCGGCCT (SEQ ID NO. 18), which is a
scrambled version used as a control.

20   Standard transfection methods were used to
produce tumor cells expressing either the bcl-2 gene or
an antisense oligodeoxynucleotide which bound to bcl-2
mRNA. It is understood that the vector could also encode
an antisense oligodeoxynucleotide which binds to bcl-2
25   pre-mRNA. The particular nucleotide sequence encoding
the antisense oligonucleotides of the invention is not
critical, except that the sequences are preferably chosen
such that they express antisense oligodeoxynucleotides
sufficient to reduce bcl-2 gene expression in tumor cells
30   and increase the sensitivity of the tumor cells to cancer
chemotherapeutic agents or sufficient to kill tumor cells
when they are treated with cancer chemotherapeutic
agents. It is only necessary that the antisense
oligodeoxynucleotide encoded in vector is expressed under

c nditions sufficient to reduce bcl-2 gene expressi n in
tumor cells.　The methods used for preparing vectors,
and, in particular, expression plasmids, for tranferring
genes into mammalian cells relies on routine techniques

5　in the field of molecular biology.　A basic text
disclosing the general methods of preparing expression
plasmids used in this invention is <u>Molecular Cloning, A
Laboratory Manual</u>, 2nd Editon, eds. Sambrook et al., Cold
Spring Harbor Laboratory Press, (1989), particularly

10　Chapter 16 on Expression of Cloned Genes in Cultured
Mammalian Cells.　Examples 15C-D below set forth
particular methods for preparing the expression plasmids
used in the present invention.　The particular vector
used to transfer the antisense oligonucleotides of the

15　present invention is not critical, and such vectors may
include vectors derived from lambda and related phages or
from filamentous phages.　It is only necessary that the
transfered nucleotide sequence encoding the antisense
oligonucleotides of the present invention be expressed in

20　the transfected tumor cell under conditions sufficient to
reduce bcl-2 gene expression in the tumor cell.　The
present invention includes expression of the antisense
oligonucleotide either from an extrachromosomal position
(e.g. from an expression plasmid) or from a position

25　integrated into the host genome itself, as mediated by
other vectors, such as recombinant retroviral vectors
(Reed et al. bcl-2 mediated tumorigenicity in a T-cell
lymphoid cell line: synergy with C-MYC and inhibition by
bcl-2 antisense. <u>PNAS</u> USA 87:3660 (1990)).

30　　　A.　　<u>Treatment of Lymphoma Cells With 18-mer
　　　　　　Synthetic bcl-2 Antisense
　　　　　　Oligodeoxynucleotides.</u>
　　　　　　Lymphoma cell line SU-DHL-4, obtained from a
use of diffuse, histiocytic, non-Hodgins lymphoma

35　(Epstein et al. Two new monoclonal antibodies (LN-1, LN-
2) reactive in B5 formalin-fixed, paraffin-embedded

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tissues with follicular center and mantl zone human B
lymphocyt s and derived tumors. J. Immunol. 133:1028 .
(1984)) and containing a t(14;18) translocation was
treated with 18-mer synthetic bcl-2-AS

5   oligodeoxynucleotides targeted for binding with the first
six codons of the bcl-2 mRNA. As a control, SU-DHL-4
cells were treated with various control oligomers,
including 18-mers having the same nucleoside composition
as the AS oligomer, but in which the bases were in

10   scrambled order (SC).

     Aliquots of 1.5 ml of HL-1 serum-free medium
(Ventrex Labs, Inc.) supplemented with 1 mM L-glutamine,
50 Units/ml penicillin, and 100 ug/ml streptomycin and
either 5 ug of purified oligonucleotides or 30 ug of

15   Lipofectin^R [1:1 w/w mixture of
N-(1-2,3-dioleyloxy)propyl)-n,n,n-trimethylammonium
chloride (DOTMA) and dioleoylphophotidylethanolamine
(DOPE)] were combined and added to 0.75 X 10^6 SU-DHL-4
cells in 3 mls of HL-1 medium. Cells were then either

20   cultured at 37°C in a humidified atmosphere of 5% CO_2/95%
air in 24 well plates (2 mls/well) for immunoblot and
RT-PCR assays, or in 96-well flat-bottom microtiter
plates (0.1 ml/well) for MTT assays. For cells in
microtiter cultures, typically 0.1 ml of additional HL-1

25   media with or without various chemotherapeutic drugs was
added after 1 day, and the cells were cultured for an
additional 2 days before performing MTT assays.

     Cells were washed once in PBS, lysed in a
buffer containing 1% Triton X100, and samples normalized

30   for protein content (25 ug) prior to size-fractionation
of proteins by SDS-PAGE (12% gels) and transfer to
nitrocellulose filters for immunoblot assays as described
in Reed et al. Cancer Res. 51:6529 (1991). Preliminary
experiments determined that aliquots of lysates

35   containing 25 ug of total protein produced results in the

linear range of the assay. Blots were first incubated
with 0.1% (v.v) of a rabbit antiserum directed against a
synthetic peptide corresponding to amino-acids (aa) 41-54
of the human Bcl-2 protein, as shown in SEQ ID NO. 21
5   (id) followed by 2.8 ug/ml biotinylated goat anti-rabbit
IgG (Vector Labs, Inc.). Bands corresponding to p26-Bcl-2
were then visualized by color development using a
Horseradish Peroxidase (HRP)-avidin-biotin complex
reagent (Vector Labs, Inc) and 3,3'-diaminobenzidine
10   (DAB). Stained blots were then incubated with a second
anti-Bcl-2 antibody directed against aa 61-76 of the
Bcl-2 protein (SEQ ID NO. 21) followed by 0.25 uCi/ml
^{125}1-protein A. Bcl-2 bands were excised from the blots
and subjected to gamma-counting.

15   Despite the mitochondrial location of Bcl-2
protein, no difference in the rate of MTT dye reduction
by mitochondrial enzymes was noted in cells that were
identical except for their levels of p26-Bcl-2. These
comparisons were made using pairs of exponentially
20   growing lymphoid cell lines that differed only in that
one line had been stably infected with a recombinant
bcl-2 retrovirus and the other with the parental
retroviral vector lacking a *bcl-2* cDNA insert (Miyashita
et al. Cancer Res. 52:5407 (1992); Blood 81:151 (1993)).

25   Anticode specific reductions in the relative
levels of bcl-2 mRNA were detected within 1 day by a
semi-quantitative reverse transcriptase polymerase chain
reaction (RT-PCR) assay. See Figure 8A.

SU-DHL-4 cells were cultured with 0.83 ug/ml of
30   oligomers complexed with 5 ug of cationic lipids
(Lipofectin; BRL/Gibco, Inc.) per ml of serum-free media
y(13,19). In Figure 8A, total RNA was isolated from
cells after 1 day and relative levels of *bcl-2* and
glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) mRNAs

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were assessed by RT-PCR assay as described in Kitada et
al. <u>Antisense R & D</u> 3:157 (1993)).

5

10

15

20

        In Figure 8(b), SU-DHL-4 cells were cultured
with pairs of either PS (squares) or PO/PS (circles) As-
and Sc-oligomers for 3 days. Relative levels of Bcl-2
protein were then measured using a quantitative
immunoblot assay, as described above, and the data
expressed as a percentage relative to cells treated with
control Sc-oligomers. The inset shows immunoblot results
for p26-Bcl-2 and a p75 cross-reactive (CR) band in a
case where As-PO/PS oligomer produced a 41% relative
decrease in Bcl-2 protein levels. In Figure 8(c), 10^{-6}M
Ara-C, MTX, or DEX was added 1 day after addition of PS
(squares) or PO/PS (circles) oligomers to cultures of
SU-DHL-4 cells, and MTT assays were performed on day 3.
Data are presented as a % control relative to cells
cultured with drugs in the absence of any oligomers, and
represent the results of 9 of 10 consecutive experiments
[in one experiment, the MTT assay failed]. Similar
results were obtained when dye exclusion assays were used
to assess cell survival rather than MTT assay [not
shown].

25

        Mean values for the data are indicated by
horizontal lines. Statistical analysis of the data was
by paired t-test (As versus Sc). Concentrations of As-
and Sc-oligomers (≈150 nM) were adjusted to maximize As
effects while maintaining sequence specificity.

30

        Variations in the amounts of starting RNA
were controlled for by RT-PCR analysis using primers
specific for GAPDH mRNA.

        The long half-life of the bcl-2 protein
(approximately 14 hours) may account for the AS-mediated
reductions in bcl-2 proteins not being as dramatic as for

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reductions in bcl-2 mRNA, taking longer to achieve (about 3 days), and appearing more variable.

Figure 8(c) shows the composite results for 10 experiments where relative levels of bcl-2 protein were compared in SU-DHL-4 cells treated with AS or SC oligomers. AS-mediated reductions in bcl-2 protein levels ranged from as much as 66% to as little as 10%, with an average relative reduction of about 30%, compared to SU-DHL-4 cells that were treated in the identical manner with control oligomers. Levels of a variety of control mitochonrial proteins such as F_1-beta-ATPase and cytochrome C, which like bcl-2 are encoded by nuclear genes, were not adversely affected by AS-oligomers (not shown), indicating that the AS-mediated reductions in bcl-2 protein levels were specific. The insert in Figure 8(b), for example, shows a comparison of p26-Bcl-2 with a 78-kDa protein that cross reacts with one of the rabbit antisera employed for immunoblot assays, demonstrating a decrease in the levels of p26-bcl-2 but not p78 in the AS-treated cells relative to cells that received control SC-oligomers.

B.   Effect of Treatment of SU-DHL-4 Cells with bcl-2 AS Oligomers on Cell Sensitivity to Cancer Chemotherapeutic Agents

This study was performed to determine whether treatment of SU-DHL-4 cells with bcl-2 AS-oligomers could increase their relative sensitivity to killing by the cancer chemotherapeutic agents Ara-C, MTX, and DEX, which are anticancer drugs.

Previous control studies demonstrated that bcl-2 AS oligomers had little or no effect on SU-DHL-4 cell growth and survival at least during the first three days of culture (Kitada et al. Antisense R & D 3:157 (1993)). AS- mediated reductions in bcl-2 protein levels in these

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lymphoma cells as well as in other cells do not typically accelerate the rate of cell death in cultures unless the cells ar deprived of serum growth factors (Reed et al. Proc. Natl. Acad. Sci. USA 87:3660 (1990)).

5   In the present work, preliminary studies demonstrated that more than 90% of SU-DHL-4 cells survived treatment for 4 days with high dose (10^{-4}) Ara-C, MTX or DEX, presumably because of their high levels of bcl-2 protein (Not shown). At these concentrations,
10   however, all drugs induced essentially complete inhibition of SU-DHL-4 cell proliferation, consistent with bcl-2 converts drugs from cytotoxic to cytostatic. Comparisons of AS and SC oligomers demonstrated that bcl-2 AS treatment markedly enhanced the sensitivity of these
15   lymphoma cells to MTX and Ara-C, and to a lesser extent to DEX (Figure 8(c)).

          Despite some variability in results, on average, the addition of bcl-2 AS oligomers to cultures of SU-DHL-4 cells treated with MTX or Ara-C resulted in
20   79-84% greater inhibition (reduction in viable cell numbers) than use of either drug alone (P< 0.002 for AS versus SC) in the absence of introducing the bcl-2 AS oligomers of the invention. Statistically significant results were obtained for DEX-treated SU-DHL-4 cells
25   (P=0.01). The 20-30% reduction in viable cell numbers observed for control oligomer-treated cells could reflect a degree of sequence non-specificity, but was probably related to the use of cationic lipids to facilitate oligomer delivery into cells.

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C.   Effect of Transfecting Cells With Expression
      Plasmids Encoding Human bcl-2 Protein on Sensitivity
      to Chemotherapeutic Agents.

            To further confirm the sequence specificity of
bcl-2 AS oligomers for enhancing sensitivity to
chemotherapeutic anticancer drugs, a study was conducted
using an Interleukin-3 (IL-3)-dependent murine
hemopoietic cell line 32D.C13 that had been stably
transfected with expression plasmid encoding either the
human bcl-2 protein or a viral homolog of bcl-2, BHRF-1,
which has only 22% homology with bcl-2. 32D.C13 cells
were obtained from Dr. Giovanni Rovera of the Wistar
Institute, Philadelphia, PA.

            Treatment of 32D cells with oligomer/cationic
lipid complexes was as described above except that 50
Units/ml of murine recombinant IL-3 (rIL-3) was included
in the HL-1 media, the initial cell density was 10^5 per
ml, and replication-defective adenovirus dl312 (MOI=200)
was added 30 minutes after exposure of cells to oligomers
to facilitate exit of DNA from endosomes [Yoshimura K, et
al. J Biol Chem. 268, 2300, (1993)].

            32D cells that had been stable transfected with
expression plasmids encoding either human p26-Bcl-2 or
EBV p19-BHRF-1 (Takayama, S. et al. submitted) were
cultured in medium (10^5/mi) containing IL-3 and PO/PS
oligomers for 3 days to achieve reductions in human Bcl-2
protein levels. The cells were then retreated with
oligomers alone (C) or in combination with various
concentrations of MTX and the relative number of viable
cells assessed by MTT assay 2 days later. Data represent
mean +/- standard deviation for triplicate determinations
and are expressed as a % relative to cells that received
no MTX. Statistical analysis of data for 10^{-6} to 10^{-4} M
MTX was by a 2-way Analysis of Variables method (Finney,
D.J. In Statistical Methods in Biological Assays, p. 72,

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1978 (3rd edition, Charles Griffin & Co., London).
Comparable results were obtained with dye exclusion
assays [not shown].

RNAs derived from the human bcl-2 construct in
32D-BCL-2 cells were a target for bcl-2 AS oligomers,
whereas RNAs from the BHRF-1 expression plasmid are not.
Thus the chemosensitivity to cytoxic drugs of 32D.C13
cells expressing BHRF-1 should have been unaffected by
the AS treatment.

Preliminary experiments demonstrated that upon
withdrawal of IL-3 from 32D.C13 cells, levels of
endogenous mouse bcl-2 protein declined and the cells
underwent apoptosis. bcl-2 and BHRF-1 comparably
supported the survival of 32D.C13 cells in the absence of
IL-3, and the proliferative rates of 32D.C13 cells
containing high levels of these proteins were similar in
the presence of IL-3, thus excluding these variables as
explanations for any differences in chemosensitivity.

Figures 9(a) and (b) compares the sensitivity
of 32D-BCL-2 and 32D-BHRF-1 cells to various
concentrations of MTX. Treatment with bcl-2 AS-oligomers
resulted in sequence-specific increases in the
sensitivity of 32D-BCL-2 cells to inhibition by MTX at
concentrations of 10^{-6} to 10^{-4} M ($P\leq 0.001$ for AS versus
SC). In contrast, treatment with bcl-2 AS oligomers
produced no significant difference in the sensitivity of
32D-BHRF-1 cells to MTX, relative to control SC-oligomers
(Figures 9(a) and (b)). These data indicate that the
effects of bcl-2 AS oligomers on chemosensitivity to
cytoxic agents drugs are sequence specific. Furthermore,
several other control oligomer, including bcl-2 sense,
other scrambled sequences with the same nucleoside
composition as AS, and oligomers with totally unrelated

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sequences all had comparatively little effect on the
chemosensitivity of the cells (Not shown).

The findings above demonstrated that bcl-2 AS
oligomers produced sequence specific reductions in bcl-2
mRNA and bcl-2 protein levels and that these events were
associated with increased sensitivity to chemotherapeutic
agents such as anticancer drugs. The portion of tumor
cells killed by the chemotherapeutic agents was greater
than the portion killed by the same amount of
chemotherapeutic agents in the absence of intoducing the
bcl-2 AS oligomers of the invention.

D.   Effect of Transfecting Cells With Expression
       Plasmids Encoding Human bcl-2 Protein on Sensitivity
       of Lymphoma Cells to Chemotherapeutic Agents.

A different strategy was employed to determine
if AS-mediated reductions in bcl-2 gene expression could
be achieved with an inducible bcl-2 AS expression plasmid
that used a heavy metal responsive human metallothionein-
IIA promoter in another translocation t(14;18)-containing
lymphoma line, RS11846. RS11846 was obtained from Dr.
Carlo Croce (Wistar Institute, Philadelphia, PA
(Tsujimoto and Croce, Proc. Natil. Acad. Sci. USA 83:5214
(1986)).

To prepare the expression plamid, a 0.91 kbp
bcl-2 cDNA (ibid)) was subcloned in either antisense (AS)
or sense (S) orientatino into a HindIII site downstream
of a human metalothionein-IIA promoter in the plasmid
pMEP-4 (Invitrogen, Inc.), which contains a hygromycin
phosphotransferase gene and the EBNA-1 gene and origin of
DNA replication from Epstein Varr Virus for high copy
episomal maintenance.

RS11846 cells (5 X 10^6) in Dulbecco's phosphate
buffered saline containing 30 ug of plasmid DNA were

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electroporated (1500 uF, 270 V/cm) using a Cellject
Electroporation System from EquiBio, Inc. Cells were
returned to their usual culture media (RPMI-L 1640
supplemented with 10% fetal bovine serum, 1 mM

5   L-glutamine, 50 Units/ml penicillin, and 100 ug/ml
streptomycin) at 2×10^5 cells per ml and cultured for
2 days before seeding cells at 2×10^5 per ml in media
containing 200 ug/ml hygromycin. After 3 weeks of
culture, the resulting bulk cell lines were passaged in

10   successively higher concentrations of hygromycin in
200 ug/ml increments until the concentration reached
1 mg/ml (about 4 weeks).

        Hygromycin-resistant RS11846 cell were cultured
in RPMI/10% serum media containing 0.5 uM $CdCl_2$ and 3 days

15   later immunoblot assays were performed using 25 ug
protein/lane essentially as described in Tanaka S, et al.
J. Biol. Chem. 268, 10920 (1993) and in Reed et al.
Cancer Res. 51:6529 (1991)).

        As summarized in Figures 10(a), (b)(1), (b)(2),

20   and (b)(3), control ("C") and bcl-2-As ("As") plasmids
were introduced into RS11846 cells and expression was
induced with either 0.5 uM $CdCl_2$ or 50 uM $ZnCl_2$ for
various times. As an additional control, RS11846 cells
containing inducible plasmids with the bcl-2 cDNA in

25   sense ("S") orientation were also analyzed. RS11846
cells were induced for 3 days and relative levels of
Bcl-2 and $F_1-\beta$-ATPase proteins were assessed by immunoblot
assay of Tanaka et al. J. Biol. Chem. 268:10920 (1993).
In Figure 10(a), RS11846 cells were cultured at 10^5

30   cells/ml in medium containing 0.5 uM $CdCl_2$ and 1 day later
10^{-7} M Ara-C or an equivalent volume of diluent control
was added. Relative numbers of viable cells were
estimated from MTT assays at various times and the mean
+/- S.D. calculated for triplicate samples. In Figures

35   10(b)(1-3), RS11846 cells were cultured as in Figure

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10(a), except that various concentrations of Ara-C, MTX,
or DEX were added. Data represent mean +/- S.D. for
triplicate samples. Statistical calculations are by
2-way Analysis of Variables. DEX served as a negative
5   control here since RS11846 cells have lost glucocorticoid
receptors.

Preliminary experiments demonstrated that
RS11846 cells tolerated the addition of up to 0.5 microM
CdCl$_2$ or to microM ZnCl$_2$ to cultures for one week,
10   experiencing a slight decrease in growth rate but
essentially no decline in percentage cell viability (Not
shown).

In the absence of heavy metal induction, the
relative levels of bcl-2 protein in RS11846 cells
15   containing the control or bcl-2 AS plasmid were
comparable, as determined by immunoblot assays (Not
shown). When 0.5 uM CdCl$_2$ or uM ZnCl$_2$ was added,
reductions in bcl-2 protein became evident in the AS-
expressing cells at 2 days and maximal inhibition of 30-
20   40% was obtained at three to four days, relative to
control RS11846 cells.

Figure 10(a) shows an example of immunoblot
data derived from RS11846 cells afaater three days of
exposure to 0.5 mM CdCl$_2$, demonstrating reduced levels of
25   bcl-2 protein in the AS-plasmid containing cells compared
to RS11846 cells that harbored the control plasmid. The
relative levels of a control mitochrondrial protein F$_1$-
beta-ATPase were comparable in all cell lines, consistent
with sequence-specific alterations in bcl-2 protein
30   levels.

When RS11846 cells containing either the
control or bcl-2-As plasmids were cultured for various
times in 0.5uM CdCl$_2$ or 50 uM ZnCl$_2$, no significant

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difference in the growth rates of these two cell lines
was observed (Figure 8(b)). Thus, As-mediated r duçtions
in *Bcl-2* protein levels by themselves did not impair
RS11846 cell proliferation or survival.

5   Inclusion of low-dose Ara-C (10^{-7}M) in the
cultures of control RS11846 cells resulted in only a
slight decline in the net numbers of viable cells,
presumably because of the high levels of Bcl-2 protein
found in these t(14;18)-containing lymphoma cells. In
10   contrast, addition of 10^{-7}M Ara-C to cultures of *bcl-2-AS*
expressing RS11846 cells was markedly inhibitory
(Figure 8(b)). Ara-C, however, had no effect on *bcl-2*
AS-expressing RS11846 cells in the absence of heavy metal
induction of the MT promoter, when directly compared with
15   RS11846 cells containing the control plasmid under the
same conditions [not shown]. Figure 8(c) shows that the
enhanced sensitivity to Ara-C observed for
bcl-2-AS-expressing RS11846 cells occurred over a wide
range of drug concentrations (P<0.001). Heavy-metal
20   induction of the *bcl-2-AS* expression plasmid also
significantly increased the relative sensitivity of
RS11846 lymphoma cells to MTX (P<0.001), but not to DEX.
Glucocorticoid receptor binding assays demonstated that
RS11846 cells have lost receptors for these steroid
25   hormones [not shown], thus providing a specificity
control showing that AS-mediated reductions in *bcl-2*
protein levels are by themselves insufficient to impair
the growth or survival of these lymphoma cells.

              Using a plurality of anticode approaches, the
30   present invention demonstrated that average reductions of
30-40% in the relative levels of bcl-2 protein markedly
enhanced the sensitivity of lymphoma cells, in
particular, t(14;18)-containing lymphoma cell lines to
chemotherapeutic agents such as conventional anticancer
35   drugs. These examples demonstrated that introducing the

claimed anticode oligomers into tumor cells achieves a reduction of bcl-2 expression and increases the chemosensitivity of ne plastic cells to chemotherapeutic agents or anticancer drugs.

5   Accordingly, the present invention achieved a method of killing tumor cells by introducing to tumor cells anticode oligomers which reduce *bcl-2* gene expression or impair Bcl-2 protein function before contacting the cells with chemotherapeutic agents

10   including anticancer drugs. The conventional anticancer drugs reduced the numbers of viable malignant cells, and the portion of tumor cells killed was greater than the portion which would have been killed by the same amount of drug in the absence of introducing the anticode

15   oligomers into the cells.

         Having thus disclosed exemplary embodiments of the present invention, it should be noted by those skilled in the art that this disclosure is exemplary only and that various other alternatives, adaptations, and

20   modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific emobodiments as illustrated herein, but is only limited by the following claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: Reed, John

   (ii) TITLE OF INVENTION: Regulation of bcl-2 Gene Expression

   (iii) NUMBER OF SEQUENCES: 23

   (iv) CORRESPONDENCE ADDRESS:
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        (C) CITY: San Diego
        (D) STATE: CA
        (E) COUNTRY: USA
        (F) ZIP: 92101-4297

   (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Diskette 3.5 inch, 1.44 Mb storage
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: US
        (B) FILING DATE:
        (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
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        (B) REGISTRATION NUMBER: 35461
        (C) REFERENCE/DOCKET NUMBER: P0041US0

   (ix) TELECOMMUNICATION INFORMATION:
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        (B) TELEFAX: (619) 236-1048

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCTTCCTAC CGCGTGCGAC   20

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(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTTCCTCT GGGAAGGATG GCGCACGCTG GGAGA   35

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCCGACCC ATCCACGTAG   20

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACGGGGTACG GAGGCTGGGT AGGTGCATCT GGT   33

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(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGACGTCC TACGGAAACA   20

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCCCAACTG CAGGATGCCT TTGTGGAACT GTACGG   36

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAAGGATG GCGCACGCTG   20

(2) INFORMATION FOR SEQ ID NO:8:

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     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: singl
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES
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    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
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CGCGTGCGAC CCTCTTG   17

(2) INFORMATION FOR SEQ ID NO:9:

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     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES
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    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
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TACCGCGTGC GACCCTC   17

(2) INFORMATION FOR SEQ ID NO:10:

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     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES
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    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
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TCCTACCGCG TGCGACC   17

(2) INFORMATION FOR SEQ ID NO:11:

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     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 bas  pairs
          (B) TYPE: nucleic acid
```

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```
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear.
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(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTTCCTACC GCGTGCG   17

(2) INFORMATION FOR SEQ ID NO:12:

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    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
```

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACCCTTCCT ACCGCGT   17

(2) INFORMATION FOR SEQ ID NO:13:

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    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
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(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAGACCCTT CCTACCG   17

(2) INFORMATION FOR SEQ ID NO:14:

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    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
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    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCGGCGGCAG CGCGG   15

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGGCGGGGCG ACGGA   15

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iv) ANTI-SENSE: YES

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGGGAGCGCG GCGGGC   16

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCTCCCAGCG TGCGCCAT   18

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 18 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGCACTCACG CTCGGCCT   18

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(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5086 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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GTGGCCCCGC CGCGCTGCCG CCGCCGCCGC TGCCAGCGAA GGTGCCGGGG CTCCGGGCCC       120

TCCCTGCCGG CGGCCGTCAG CGCTCGGAGC GAACTGCGCG ACGGGAGGTC CGGGAGGCGA       180

CCGTAGTCGC GCCGCCGCGC AGGACCAGGA GGAGGAGAAA GGGTGCGCAG CCCGGAGGCG       240

GGGTGCGCCG GTGGGGTGCA GCGGAAGAGG GGGTCCAGGG GGGAGAACTT CGTAGCAGTC       300

ATCCTTTTTA GGAAAAGAGG GAAAAAATAA AACCCTCCCC CACCACCTCC TTCTCCCCAC       360

CCCTCGCCGC ACCACACACA GCGCGGGCTT CTAGCGCTCG GCACCGGCGG GCCAGGCGCG       420

TCCTGCCTTC ATTTATCCAG CAGCTTTTCG GAAAATGCAT TTGCTGTTCG GAGTTTAATC       480

AGAAGACGAT TCCTGCCTCC GTCCCCGGCT CCTTCATCGT CCCATCTCCC CTGTCTCTCT       540

CCTGGGGAGG CGTGAAGCGG TCCCGTGGAT AGAGATTCAT GCCTGTGTCC GCGCGTGTGT       600

GCGCGCGTAT AAATTGCCGA GAAGGGGAAA ACATCACAGG ACTTCTGCGA ATACCGGACT       660

GAAAATTGTA ATTCATCTGC CGCCGCCGCT GCCAAAAAAA AACTCGAGCT CTTGAGATCT       720

CCGGTTGGGA TTCCTGCGGA TTGACATTTC TGTGAAGCAG AAGTCTGGGA ATCGATCTGG       780

AAATCCTCCT AATTTTTACT CCCTCTCCCC CCGACTCCTG ATTCATTGGG AAGTTTCAAA       840

TCAGCTATAA CTGGAGAGTG CTGAAGATTG ATGGGATCGT TGCCTTATGC ATTTGTTTTG       900

GTTTTACAAA AAGGAAACTT GACAGAGGAT CATGCTGTAC TTAAAAAATA CAAGTAAGTC       960

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AGTGCATTCG AGTAAATTTA ATTTCCAGGC AGCTTAATAC ATTGTTTTTA GCCGTGTTAC      1080

TTGTAGTGTG TATGCCCTGC TTTCACTCAG TGTGTACAGG GAAACGCACC TGATTTTTTA      1140

CTTATTAGTT TGTTTTTTCT TTAACCTTTC AGCATCACAG AGGAAGTAGA CTGATATTAA      1200

CAATACTTAC TAATAATAAC GTGCCTCATG AAATAAAGAT CCGAAAGGAA TTGGAATAAA      1260

AATTTCCTGC GTCTCATGCC AAGAGGGAAA CACCAGAATC AAGTGTTCCG CGTGATTGAA      1320
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GACACCCCCT CGTCCAAGAA TGCAAAGCAC ATCCAATAAA ATAGCTGGAT TATAACTCCT   1380
CTTCTTTCTC TGGGGGCCGT GGGGTGGGAG CTGGGGCGAG AGGTGCCGTT GGCCCCCGTT   1440
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GTGATGAAGT ACATCCATTA TAAGCTGTCG CAGAGGGGCT ACGAGTGGGA TGCGGGAGAT   1560
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CTGTCTCTGA AGACTCTGCT CAGTTTGGCC CTGGTGGGAG CTTGCATCAC CCTGGGTGCC   2160
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GGAAAGTATT TTTTTAAGCT ACCAATTGTG CCGAGAAAG CATTTTAGCA ATTTATACAA   2940
TATCATCCAG TACCTTAAAC CCTGATTGTG TATATTCATA TATTTTGGAT ACGCACCCCC   3000
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```
CAACTCCCAA TACTGGCTCT GTCTGAGTAA GAAACAGAAT CCTCTGGAAC TTGAGGAAGT   3060
GAACATTTCG GTGACTTCCG ATCAGGAAGG CTAGAGTTAC CCAGAGCATC AGGCCGCCAC   3120
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TGTGGTCTCC GAATGTCTGG AAGCTGATGG ATGGAGCTCA GAATTCCACT GTCAAGAAAG   3300
AGCAGTAGAG GGGTGTGGCT GGGCCTGTCA CCCTGGGGCC CTCCAGGTAG GCCCGTTTTC   3360
ACGTGGAGCA TAGGAGCCAC GACCCTTCTT AAGACATGTA TCACTGTAGA GGGAAGGAAC   3420
AGAGGCCCTG GGCCTTCCTA TCAGAAGGAC ATGGTGAAGG CTGGGAACGT GAGGAGAGGC   3480
AATGGCCACG GCCCATTTTG GCTGTAGCAC ATGGCACGTT GGCTGTGTGG CCTTGGCCAC   3540
CTGTGAGTTT AAAGCAAGGC TTTAAATGAC TTTGGAGAGG GTCACAAATC CTAAAAGAAG   3600
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ACCTAAGAAA AACCTGGATG TCACTGGCCA CTGAGGAGCT TTGTTTCAAC CAAGTCATGT   4200
GCATTTCCAC GTCAACAGAA TTGTTTATTG TGACAGTTAT ATCTGTTGTC CCTTTGACCT   4260
TGTTTCTTGA AGGTTTCCTC GTCCCTGGGC AATTCCGCAT TTAATTCATG GTATTCAGGA   4320
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GACCAGCAGA TTCAAATCTA TGGTGGTTTG ACCTTTAGAG AGTTGCTTTA CGTGGCCTGT   4440
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GTCCTTCAGG GTCTTCCTGA AATGCAGTGG TCGTTACGCT CCACCAAGAA AGCAGGAAAC   4560
CTGTGGTATG AAGCCAGACC TCCCCGGCGG GCCTCAGGGA ACAGAATGAT CAGACCTTTG   4620
AATGATTCTA ATTTTTAAGC AAAATATTAT TTTATGAAAG GTTTACATTG TCAAAGTGAT   4680
```

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```
GAATATGGAA TATCCAATCC TGTGCTGCTA TCCTGCCAAA ATCATTTTAA TGGAGTCAGT      4740

TTGCAGTATG CTCCACGTGG TAAGATCCTC CAAGCTGCTT TAGAAGTAAC AATGAAGAAC      4800

GTGGACGTTT TTAATATAAA GCCTGTTTTG TCTTTTGTTG TTGTTCAAAC GGGATTCACA      4860

GAGTATTTGA AAAATGTATA TATATTAAGA GGTCACGGGG GCTAATTGCT AGCTGGCTGC      4920

CTTTTGCTGT GGGGTTTTGT TACCTGGTTT TAATAACAGT AAATGTGCCC AGCCTCTTGG      4980

CCCCAGAACT GTACAGTATT GTGGCTGCAC TTGCTCTAAG AGTAGTTGAT GTTGCATTTT      5040

CCTTATTGTT AAAAACATGT TAGAAGCAAT GAATGTATAT AAAAGC                     5086
```

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(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 717 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 1..717

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC CGG GAG ATA GTG ATG        48
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
1               5                  10                  15

AAG TAC ATC CAT TAT AAG CTG TCG CAG AGG GGC TAC GAG TGG GAT GCG        96
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
                20                  25                  30

GGA GAT GTG GGC GCC GCG CCC CCG GGG GCC GCC CCC GCA CCG GGC ATC       144
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
            35                  40                  45

TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA GCC GCA TCC CGC GAC       192
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
        50                  55                  60

CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG GCT GCC CCC GGC GCC       240
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
65                  70                  75                  80

GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG GTC CAC CTG GCC       288
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
                85                  90                  95

CTC CGC CAA GCC GGC GAC GAC TTC TCC CGC CGC TAC CGC GGC GAC TTC       336
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
                100                 105                 110

GCC GAG ATG TCC AGC CAG CTG CAC CTG ACG CCC TTC ACC GCG CGG GGA       384
Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
            115                 120                 125

CGC TTT GCC ACG GTG GTG GAG GAG CTC TTC AGG GAC GGG GTG AAC TGG       432
Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
        130                 135                 140
```

```
GGG AGG ATT GTG GCC TTC TTT GAG TTC GGT GGG GTC ATG TGT GTG GAG          480
Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145                 150                 155                 160

AGC GTC AAC CGG GAG ATG TCG CCC CTG GTG GAC AAC ATC GCC CTG TGG          528
Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
                    165                 170                 175

ATG ACT GAG TAC CTG AAC CGG CAC CTG CAC ACC TGG ATC CAG GAT AAC          576
Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
                180                 185                 190

GGA GGC TGG GAT GCC TTT GTG GAA CTG TAC GGC CCC AGC ATG CGG CCT          624
Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
            195                 200                 205

CTG TTT GAT TTC TCC TGG CTG TCT CTG AAG ACT CTG CTC AGT TTG GCC          672
L u Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
210                 215                 220

CTG GTG GGA GCT TGC ATC ACC CTG GGT GCC TAT CTG AGC CAC AAG              717
Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
225                 230                 235
```

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(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 239 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
 1           5               10              15

Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
            20              25              30

Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
        35              40              45

Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
    50              55              60

Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
 65              70              75              80

Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
            85              90              95

Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
            100             105             110

Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
        115             120             125

Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
    130             135             140

Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145             150             155             160

Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
            165             170             175

Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
        180             185             190

Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
        195             200             205

Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
    210             215             220
```

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Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
225   230   235

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 615 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 1..615

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC CGG GAG ATA GTG ATG      48
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
 1               5                  10                  15

AAG TAC ATC CAT TAT AAG CTG TCG CAG AGG GGC TAC GAG TGG GAT GCG      96
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
                20                  25                  30

GGA GAT GTG GGC GCC GCG CCC CCG GGG GCC GCC CCC GCA CCG GGC ATC     144
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
                35                  40                  45

TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA GCC GCA TCC CGC GAC     192
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
            50                  55                  60

CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG GCT GCC CCC GGC GCC     240
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
65                  70                  75                  80

GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG GTC CAC CTG GCC     288
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
                85                  90                  95
```

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```
GTC CGC CAA GCC GGC GAC GAC TTC TCC CGC CGC TAC CGC GGC GAC TTC     336
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
            100                 105                 110

GCC GAG ATG TCC AGC CAG CTG CAC CTG ACG CCC TTC ACC GCG CGG GGA     384
Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
            115                 120                 125

CGC TTT GCC ACG GTG GTG GAG GAG CTC TTC AGG GAC GGG GTG AAC TGG     432
Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
            130                 135                 140

GGG AGG ATT GTG GCC TTC TTT GAG TTC GGT GGG GTC ATG TGT GTG GAG     480
Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145                 150                 155                 160

AGC GTC AAC CGG GAG ATG TCG CCC CTG GTG GAC AAC ATC GCC CTG TGG     528
Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
                165                 170                 175

ATG ACT GAG TAC CTG AAC CGG CAC CTG CAC ACC TGG ATC CAG GAT AAC     576
Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
                180                 185                 190

GGA GGC TGG GTA GGT GCA TCT GGT GAT GTG AGT CTG GGC                 615
Gly Gly Trp Val Gly Ala Ser Gly Asp Val Ser Leu Gly
                195                 200                 205
```

(2) INFORMATION FOR SEQ ID NO:23:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 205 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
1               5                   10                  15

Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
                20                  25                  30

Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
            35                  40                  45

Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
    50                  55                  60

Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
65                  70                  75                  80
```

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Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala
                85   90   95

Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe
              100   105   110

Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
        115   120   125

Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
    130   135   140

Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145   150   155   160

Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
            165   170   175

Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
            180   185   190

Gly Gly Trp Val Gly Ala Ser Gly Asp Val Ser Leu Gly
        195   200   205

CLAIMS:

1.   An anticode oligomer which binds to a site
in mRNA transcribed from the human bcl-2 gen , said site
being a strategic site selected from the splice donor
site, the splice acceptor site and the or a portion of
5   the 5'-untranslated region of said mRNA, which anticode
oligomer, when brought into contact with cancer cells
expressing the human bcl-2 gene, has the property of
reducing the expression of at least one bcl-2 gene
product and thereby inducing programmed cell death of
10   said cancer cells.

2.   An anticode oligomer as claimed in claim 1
wherein said anticode oligomer is an antisense
oligonucleotide or analogue thereof.

3.   An anticode oligomer as claimed in claim 1
15   or claim 2 wherein said anticode oligomer is
substantially complementary to said strategic site.

4.   An anticode oligomer as claimed in any one
of the preceding claims wherein said strategic site is in
the 5'-untranslated region of said mRNA expressed from
20   the human bcl-2 gene and wherein said anticode oligomer
binds exclusively to the or a portion of the 5'-
untranslated region.

5.   An anticode oligomer as claimed in any one
of claims 2 to 4 wherein said anticode oligomer is a
25   phosphodiester/methylphosphonate chimera or a
phosphodiester/phosphorothioate chimera or a
methylphosphonate/phosphorothioate chimera.

6.   An anticode oligomer as claimed in any one
of claims 2 to 4 wherein said anticode oligomer or
30   chirally enriched form thereof is comprised of one or
more groups selected from methylphosphonates,

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phosphorothioates, diesters, phorphorodithioates, or 2'
substituted derivatives.

     7.   An anticode oligomer as claimed in any one
of the preceding claims which is two to two hundred
nucleotide bases long.

     8.   An anticode oligomer as claimed in any one
of the preceding claims which is ten to forty nucleotide
bases long.

     9.   An anticode oligomer as claimed in any one
of the preceding claims wherein the cells expressing the
human bcl-2 gene are human tumor cells.

     10.   An anticode oligomer as claimed in claim 8
wherein the strategic site is a sequence portion in pre-
mRNA expressed from said bcl-2 gene.

     11.   An anticode oligomer as claimed in claim
10 wherein said anticode oligomer is substantially
complementary to and binds to a strategic site in said
pre-mRNA.

     12.   An anticode oligomer as claimed in claim
10 or claim 11 wherein said strategic site in said pre-
mRNA is selected from the translation initiation site,
the splice donor site, the splice acceptor site, codons 1
to 6 of the bcl-2 open reading frame and the or a portion
of the 5'-untranslated region.

     13.   An anticode oligomer as claimed in any one
of claims 9 to 12 which is two to two hundred nucleotide
bases long.

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14. An anticode oligomer as claimed in any one
of claims 9-13 which is ten to forty nucleotide bases
long.

15. An anticode oligomer as claimed in any one
of claims 9 to 13 wherein the cells expressing the human
bcl-2 gene are human tumor cells.

16. An anticode oligomer useful for inhibiting
the growth of cells expressing the human bcl-2 gene which
anticode oligomer binds with a strategic site of RNA
expressed from the human bcl-2 gene, which anticode
oligomer is a phosphodiester/methylphosphonate chimera or
a phosphodiester/phosphorothioate chimera or a
methylphosphonate/phosphorothioate chimera of an
antisense oligonucleotide which when brought into contact
with cancer cells expressing the human bcl-2 gene, has
the property of reducing the expression of at least one
bcl-2 gene product and thereby inducing programmed cell
death of said cancer cells.

17. An anticode oligomer useful for inhibiting
the growth of cells expressing the human bcl-2 gene
comprising an anticode oligomer which binds with a
strategic site of RNA expressed from the human bcl-2
gene, which anticode oligomer or chirally enriched form
thereof is comprised of one or more groups selected from
methylphosphonates, phosphorothioates, diesters,
phorphorodithioates, or 2' substituted derivatives, which
anticode oligomer when brought into contact with cancer
cells expressing the human bcl-2 gene, has the property
of reducing the expression of at least one bcl-2 gene
product and thereby inducing programmed cell death of
said cancer cells.

SUBSTITUTE SHEET (RULE 26)

18. A vector for transfecting human cancer cells comprising a nucleotide sequence that encodes an anticode oligomer as claim d in any one of claims 1 to 4.

19. A vector for transfecting human cancer cells comprising a nucleotide sequence that encodes an anticode oligomer which reduces expression from the human bcl-2 gene in said cells, said vector being derived from a phage.

20. A composition useful for inhibiting the growth of cells expressing the human bcl-2 gene which comprises an anticode oligomer as claimed in any one of claims 1 to 17 together with a pharmaceutically acceptable carrier.

21. A composition useful for inhibiting the growth of cells expressing the human bcl-2 gene which comprises a vector as claimed in claim 18 or claim 19 and a pharmaceutically acceptable carrier.

22. A composition as claimed in claim 20 wherein said pharmaceutically acceptable carrier is a cationic lipid.

23. A composition as claimed in claim 20 wherein said anticode oligomer is encapsulated in liposomes.

24. Use of an anticode oligomer as claimed in any one of claims 1 to 17 in the manufacture of a medicament for treating human cancer.

25. Use of a vector as claimed in claim 18 or claim 19 in the manufacture of a medicament for treating human cancer.

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26. Use of an anticode oligomer which binds
with a sequence portion of mRNA expressed from the human
bcl-2 g ne in the manufacture of a medicament for
treating solid tumors in humans.

5   27. Use of an anticode oligomer which binds
with a sequence portion of mRNA expressed from the human
bcl-2 gene in the manufacture of a medicament for
treating breast cancer.

28. Materials for simultaneous or sequential
10   use for killing human cancer cells expressing the bcl-2
gene comprising:

          an anticode oligomer which binds to a
          sequence portion of mRNA expressed from a
          human bcl-2 gene and;

15   a chemotherapeutic agent toxic to said
          human cancer cells.

29. Materials as claimed in claim 28 wherein
said chemotherapeutic agent is selected from
antimetabolites, alkylating agents, plant alkaloids and
20   antibiotics.

30. Materials as claimed in claim 29 wherein
said chemotherapeutic agent further comprises a
radionuclide.

31. Materials as claimed in claim 28 or claim
25   29 wherein said anticode oligomer is as claimed in any
one of claims 1 to 17.

82.   A method of inducing programmed cell
death of cancer cells which express the human bcl-2 gene
comprising the steps of:

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a. providing an antic de oligomer as
    claimed in any one of claims 1 to I7;
    and

b. contacting said cel}s with said
    anticode oligomer under conditions
    sufficient to induce programmed cell
    death in said cells.

33. A method of killing human cancer cells
which express the human bcl-2 gene comprising the steps
of:

a. providing an anticode oligomer which
    binds with a sequence portion of mRNA
    expressed from the human bcl-2 gene;

b. contacting said cells with said
    anticode oligomer under conditions to
    inhibit growth of said cells; and

c. contacting said cells with an amount
    of at least one secondary therapeutic
    agent toxic to said cells sufficient
    to kill at least a portion thereof.

34. A method as claimed in claim 33 wherein
said anticode oligomer is as claimed in any one of claims
1 to 17.

35. A method as claimed in claim 33 wherein
said secondary agent is a chemotherapeutic agent.

36. A method as claimed in any one of claims
33-35 wherein said chemotherapeutic agent is selected
from antimetabolites, alkylating agents, plant alkaloids
and antibiotics.

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37.   A method as claimed in claims 33 to 34
wherein said sec ndary therapeutic agent is a
radiotherapeutic agent.

38.   A method as claimed in claims 33, 34, or
5   37 in which said step of contacting said cells with a
radiotherapeutic agent comprises contacting said cells
with a beam of radiation, wherein said radiation
comprises either particle radiation or non-particle
radiation generated from a source which is non-contiguous
10   with said cells.

39.   A method as claimed in claims 33, 34, or
37 in which said step of contacting said cells with a
radiotherapeutic agent comprises contacting said cells
with a radionuclide.

15   40.   A composition useful for killing human
cancer cells expressing the bcl-2 gene which comprises an
anticode oligomer as claimed in any one of claims 1-17
and a chemotherapeutic agent toxic to said human cancer
cells.

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FIG. 1 (a)
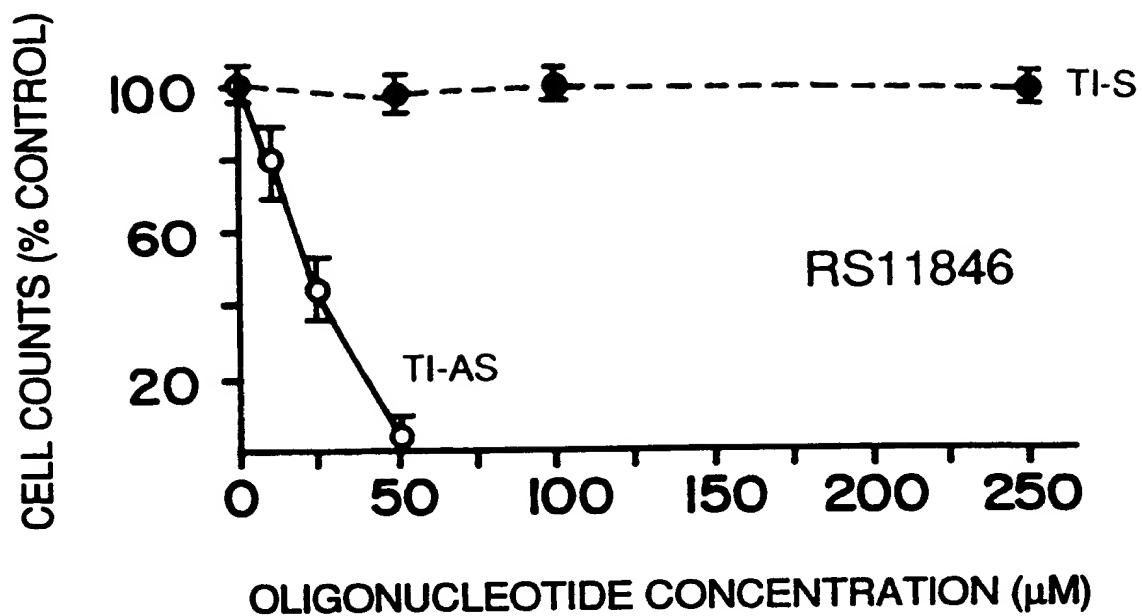
FIG. 1 (b)
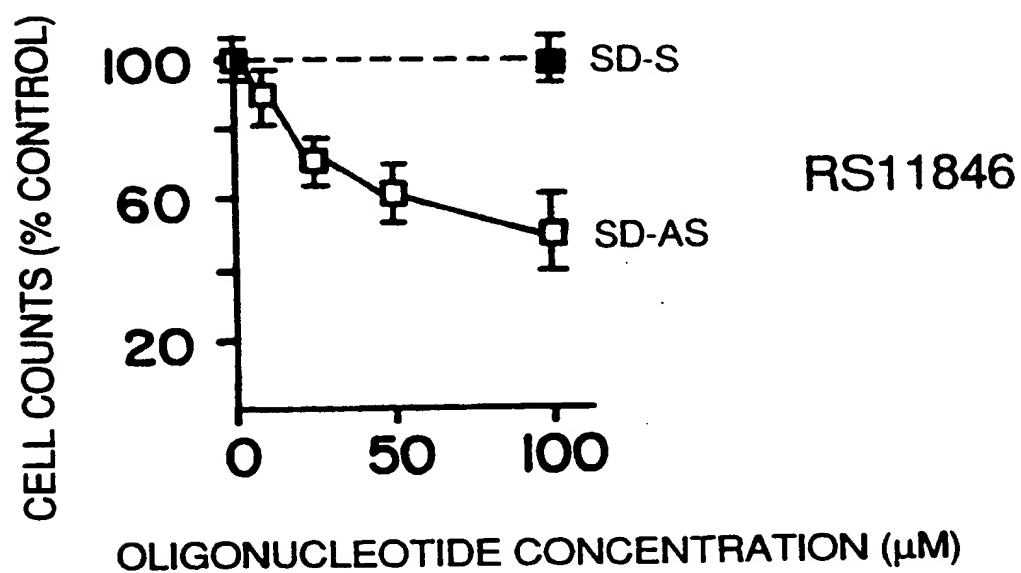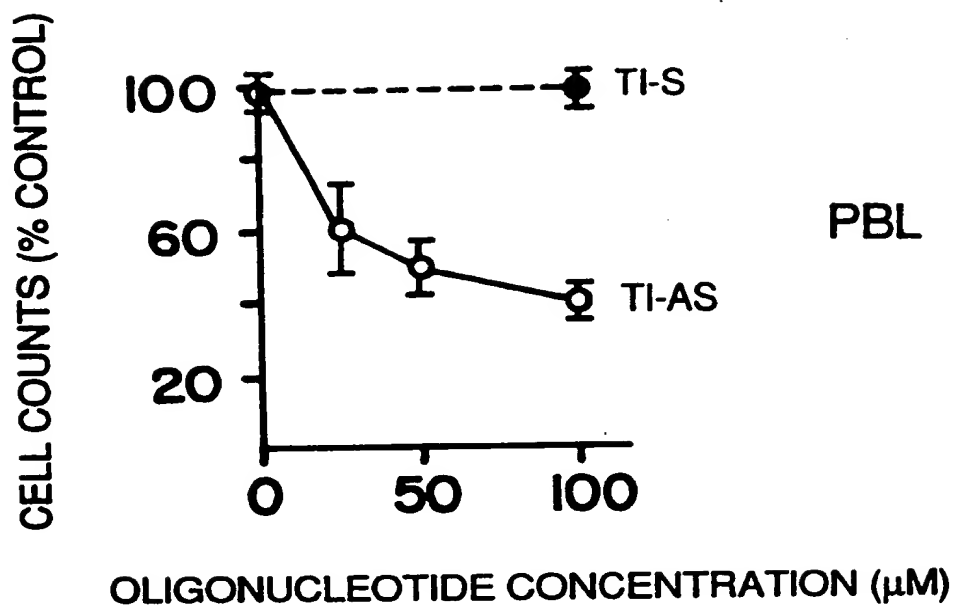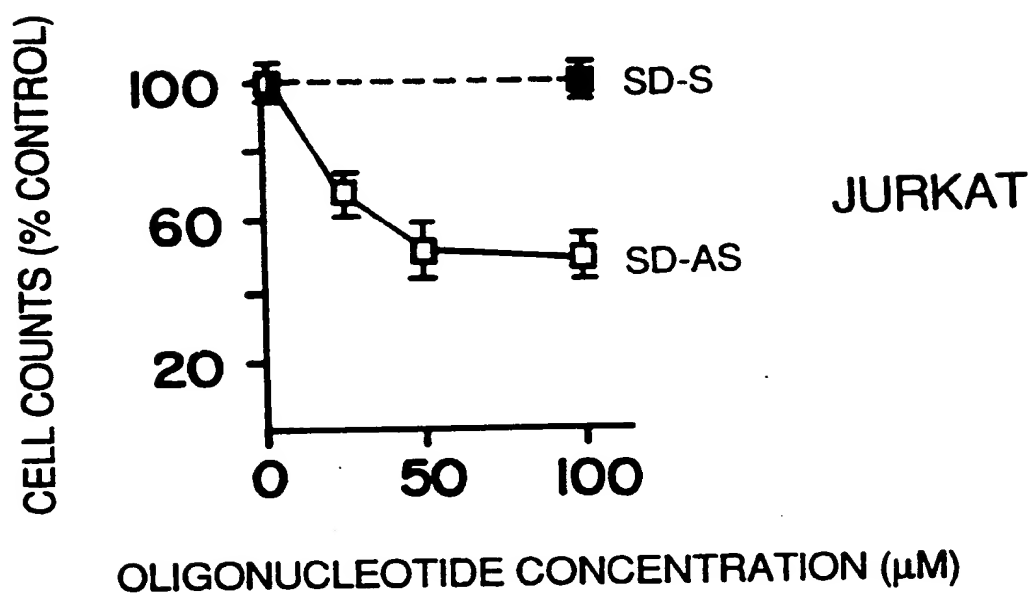
FIG. 1(c)

FIG. 1(d)

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FIG. 2(a).
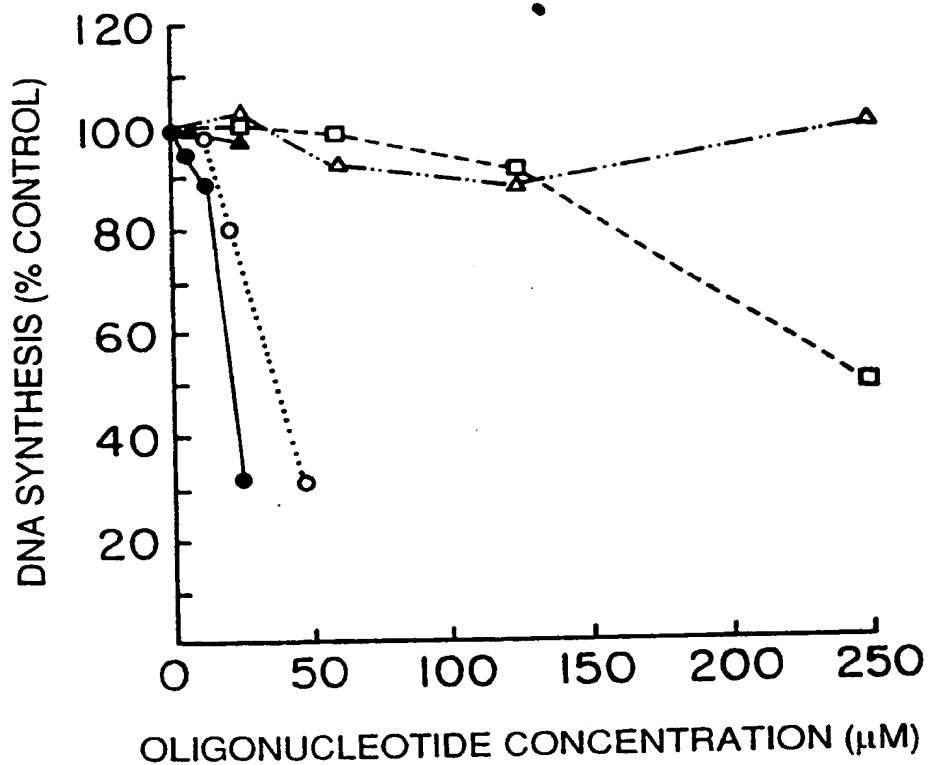
FIG. 2(b)
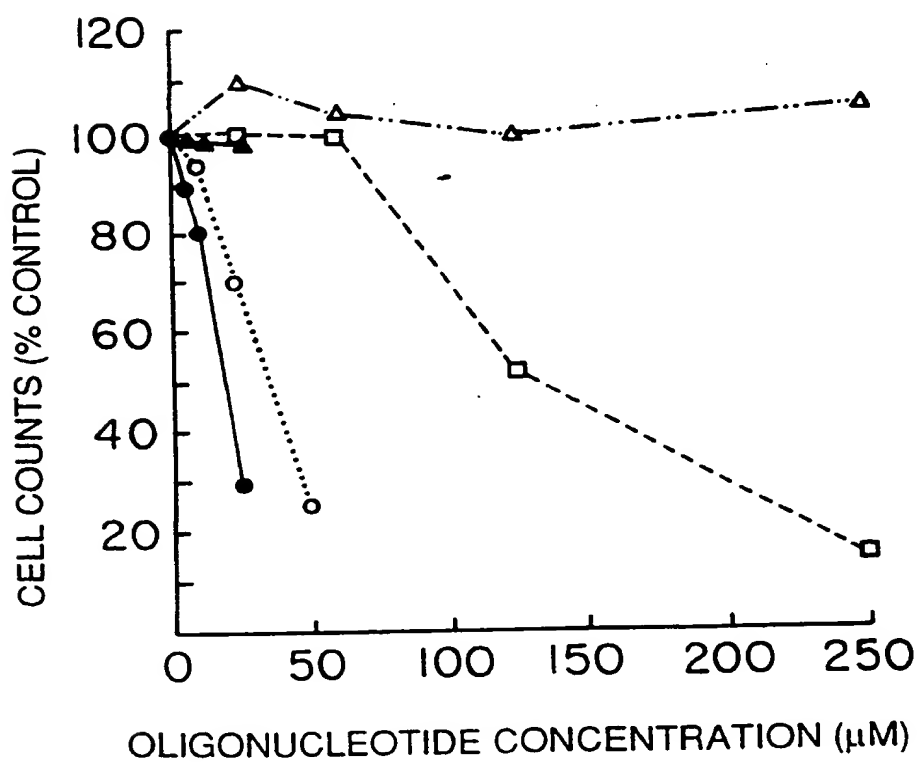
FIG. 3

FIG. 4(a)

FIG. 4(b)

F I G. 5

FIG. 6 (a)

OLIGODEOXYNUCLEOTIDE
CONCENTRATION (µM)

FIG. 6(b)

FIG. 7.(a)

FIG. 7(b)

FIG. 7(c)

Relative Cell Number

| 40 | 60 | 120 | 160 | 200

Fluorescence

FIG. 7(d)

Relative Cell Number

| 40 | 80 | 120 | 160 | 200

Fluorescence

FIG. 8A

FIG. 8B

FIG. 8C

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FIG. 9(a)

FIG. 9(b)

% of Control (Mean ± S.E.)

FIG. IOA

FIG. IOB (I)

FIG. IOB(2)

FIG. 10B (3)

Cells / well (Mean ± S.E.) (x 10^{-4})

Log - [Drug Concentration] [M]

FIG. 11

FIG. 12

FIG. 13(a)

ATG oligo
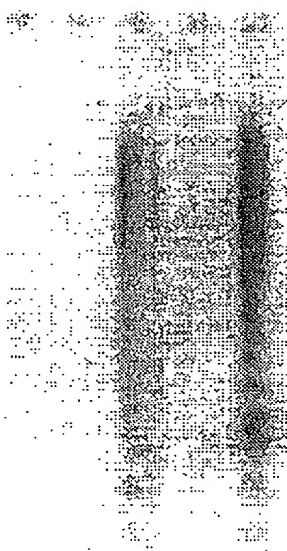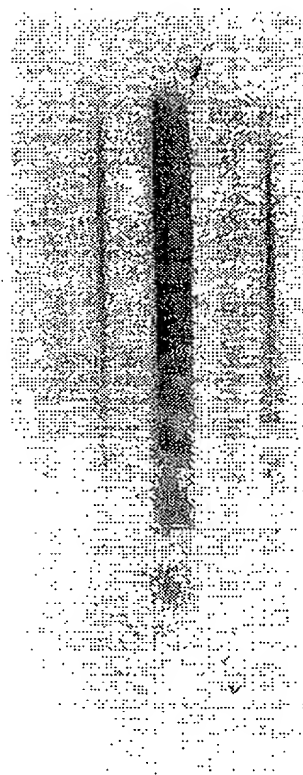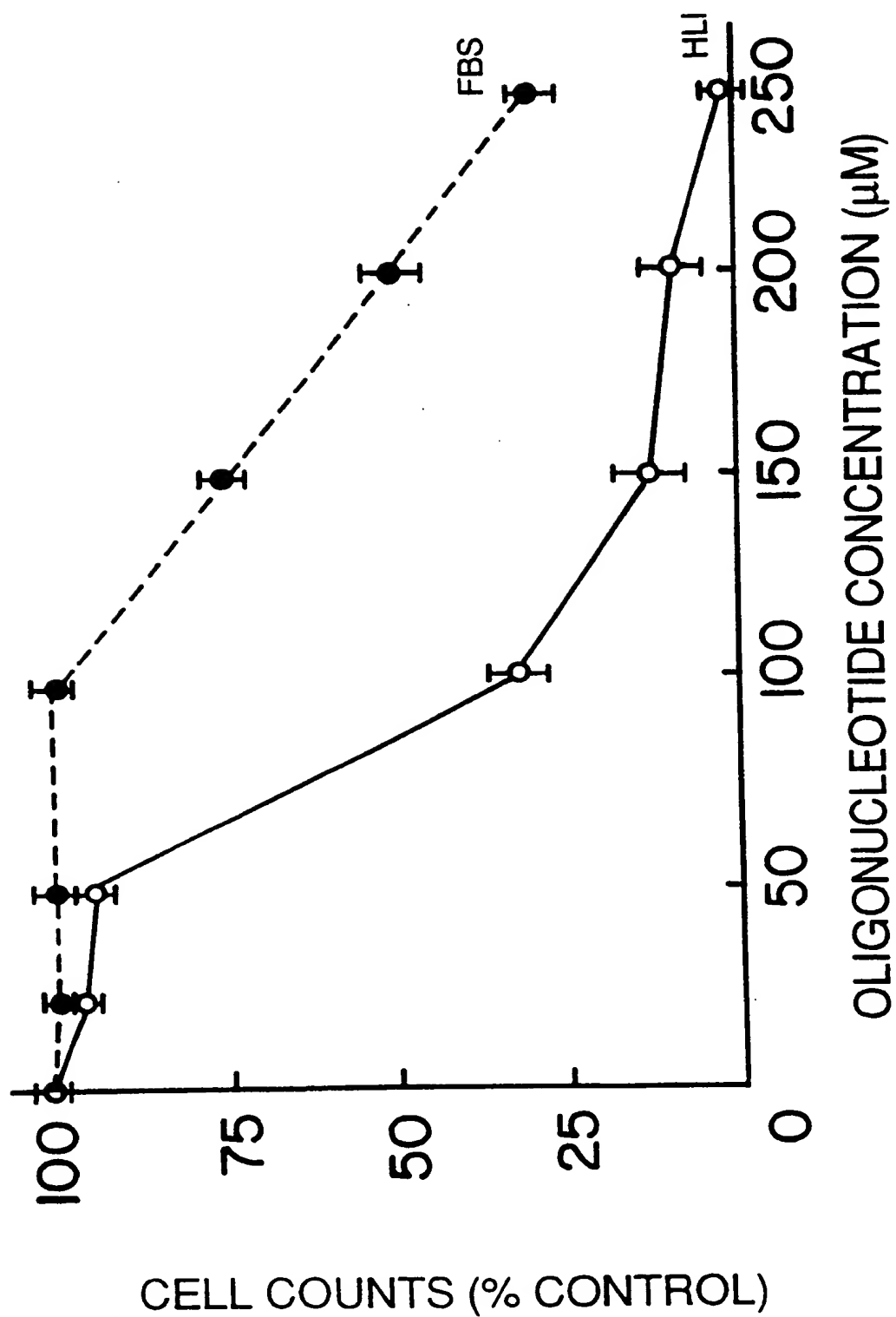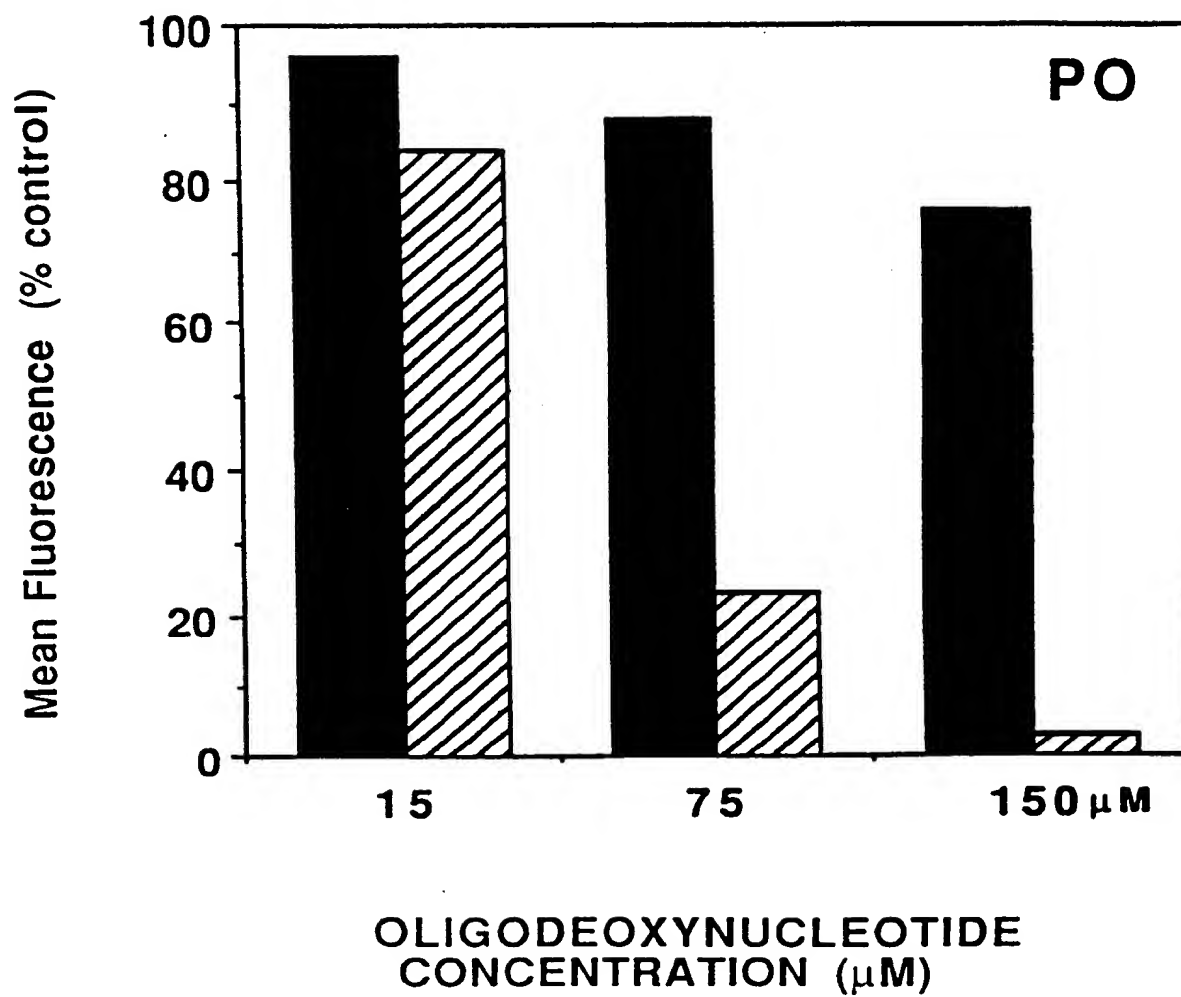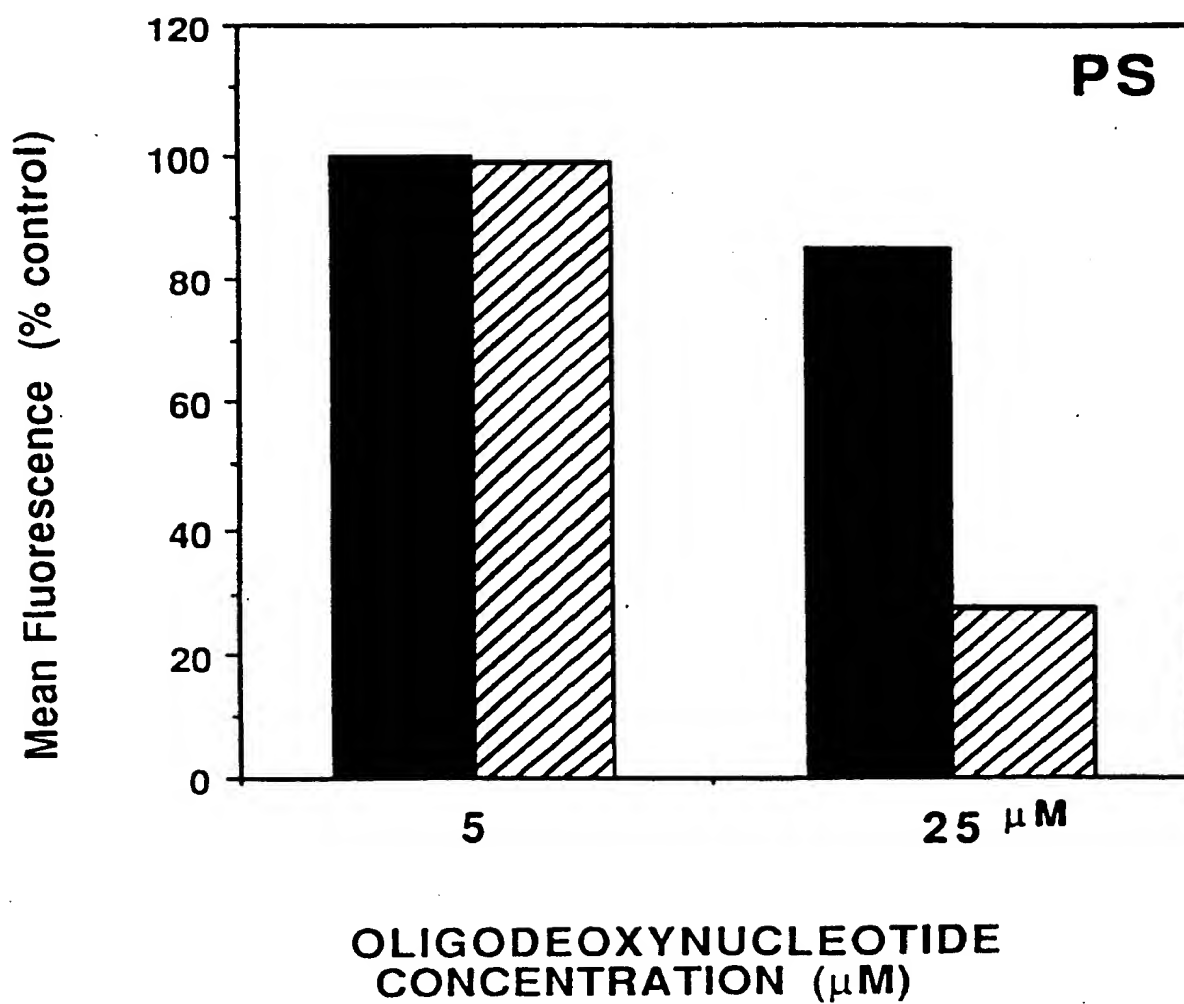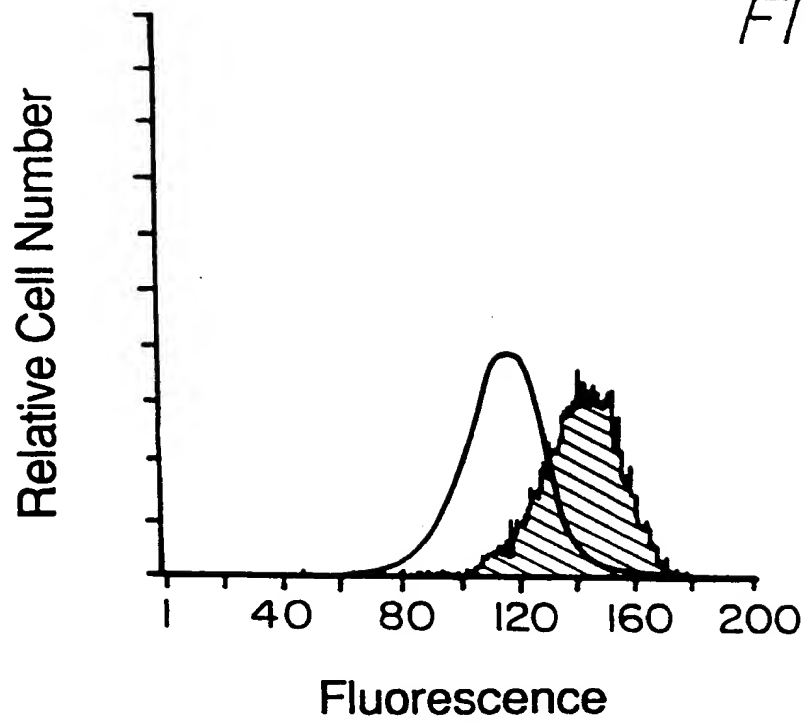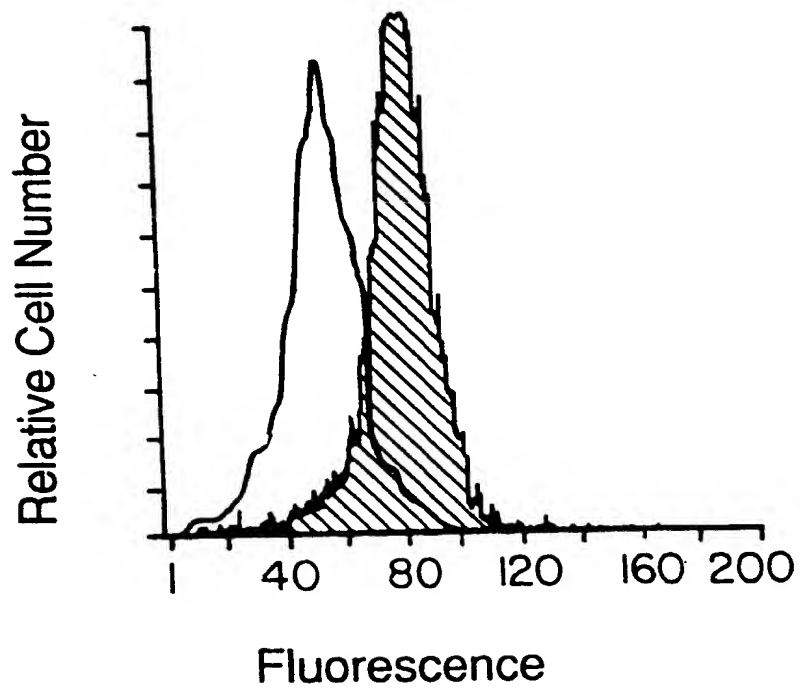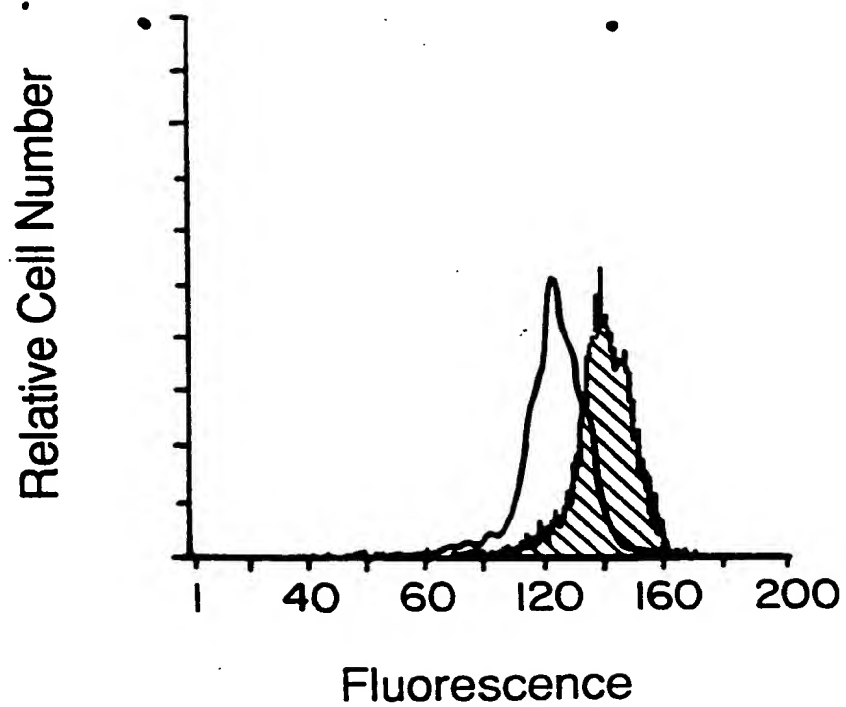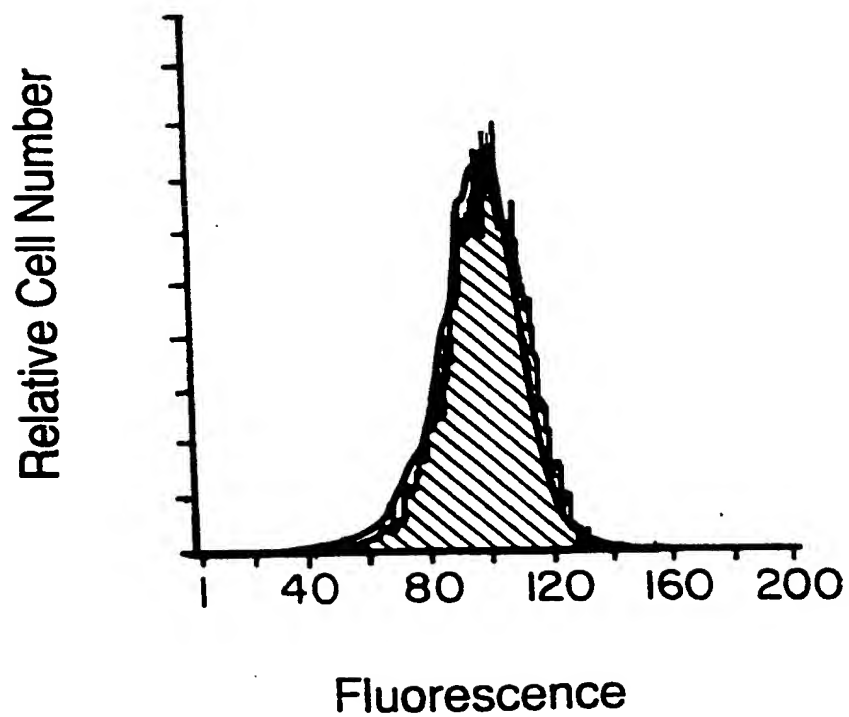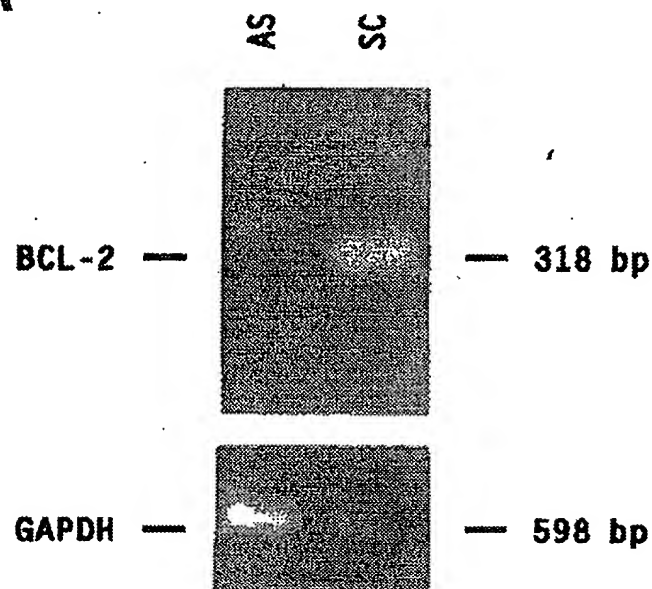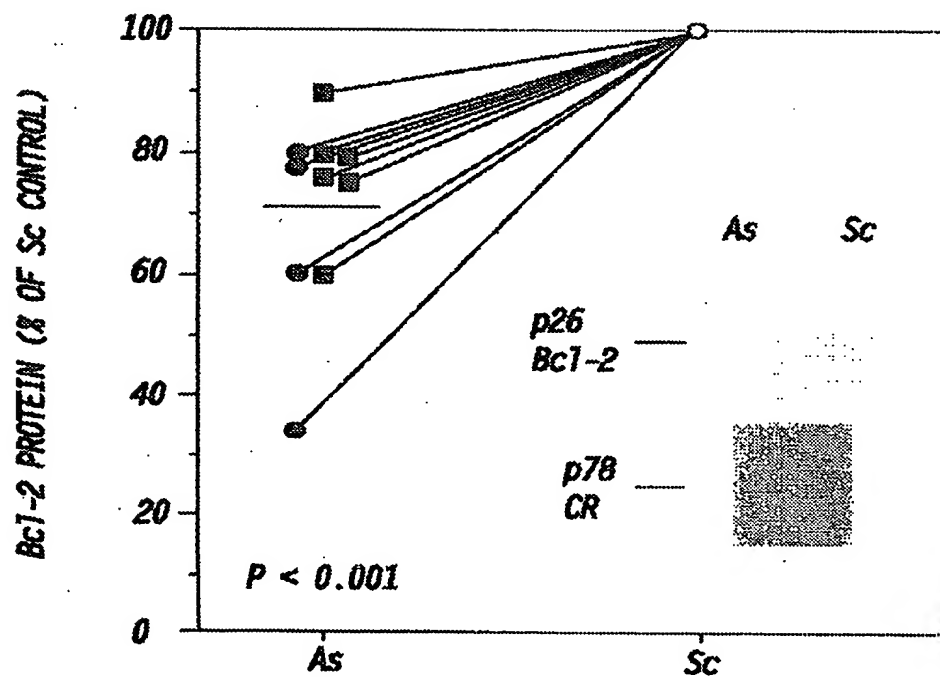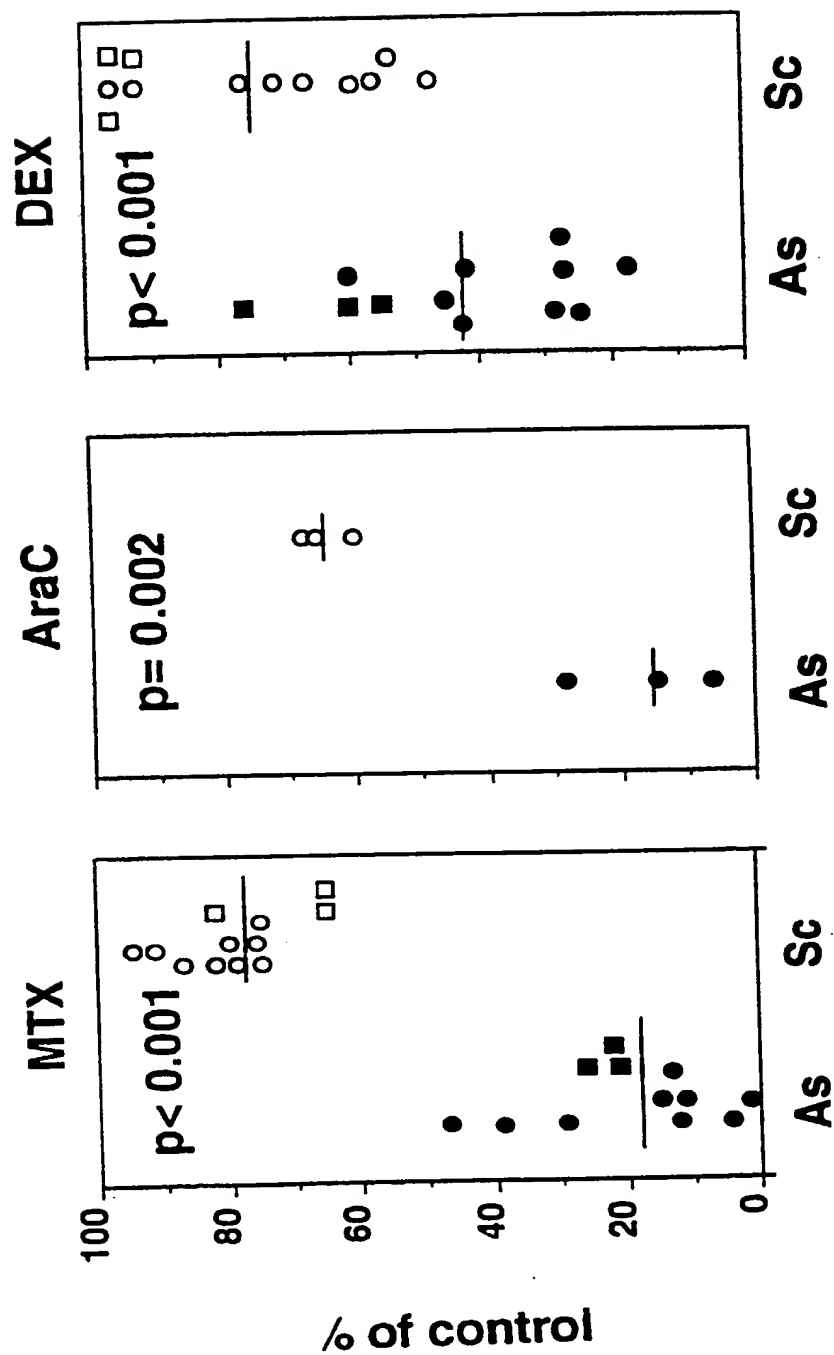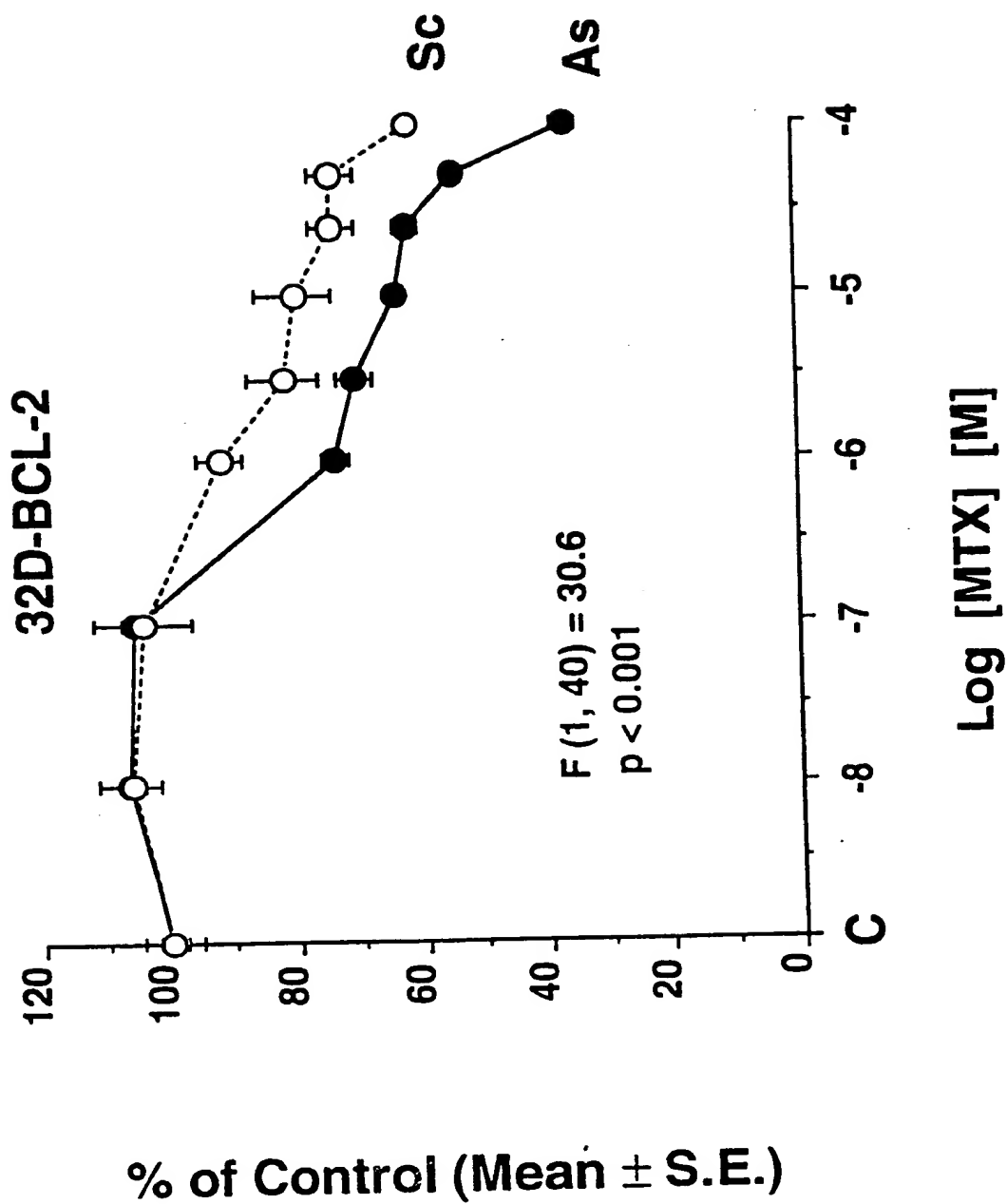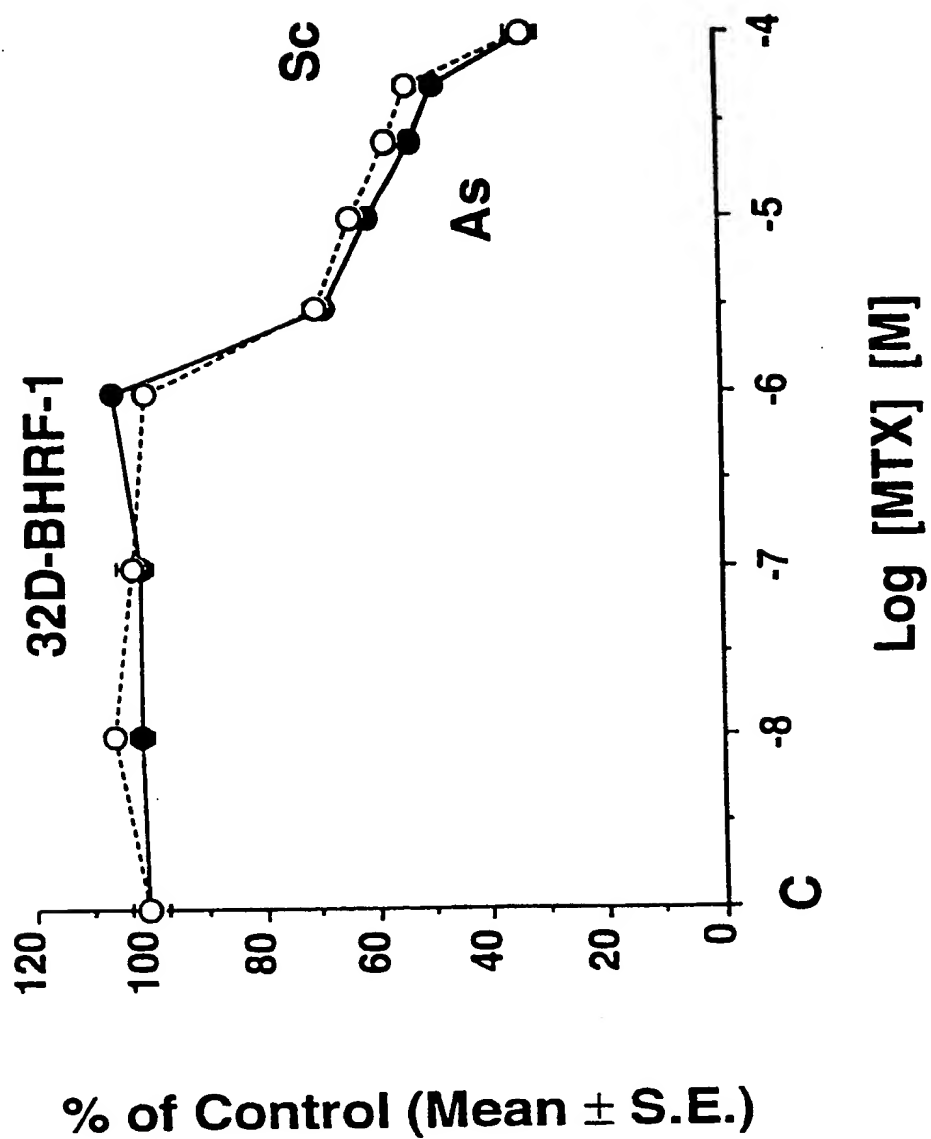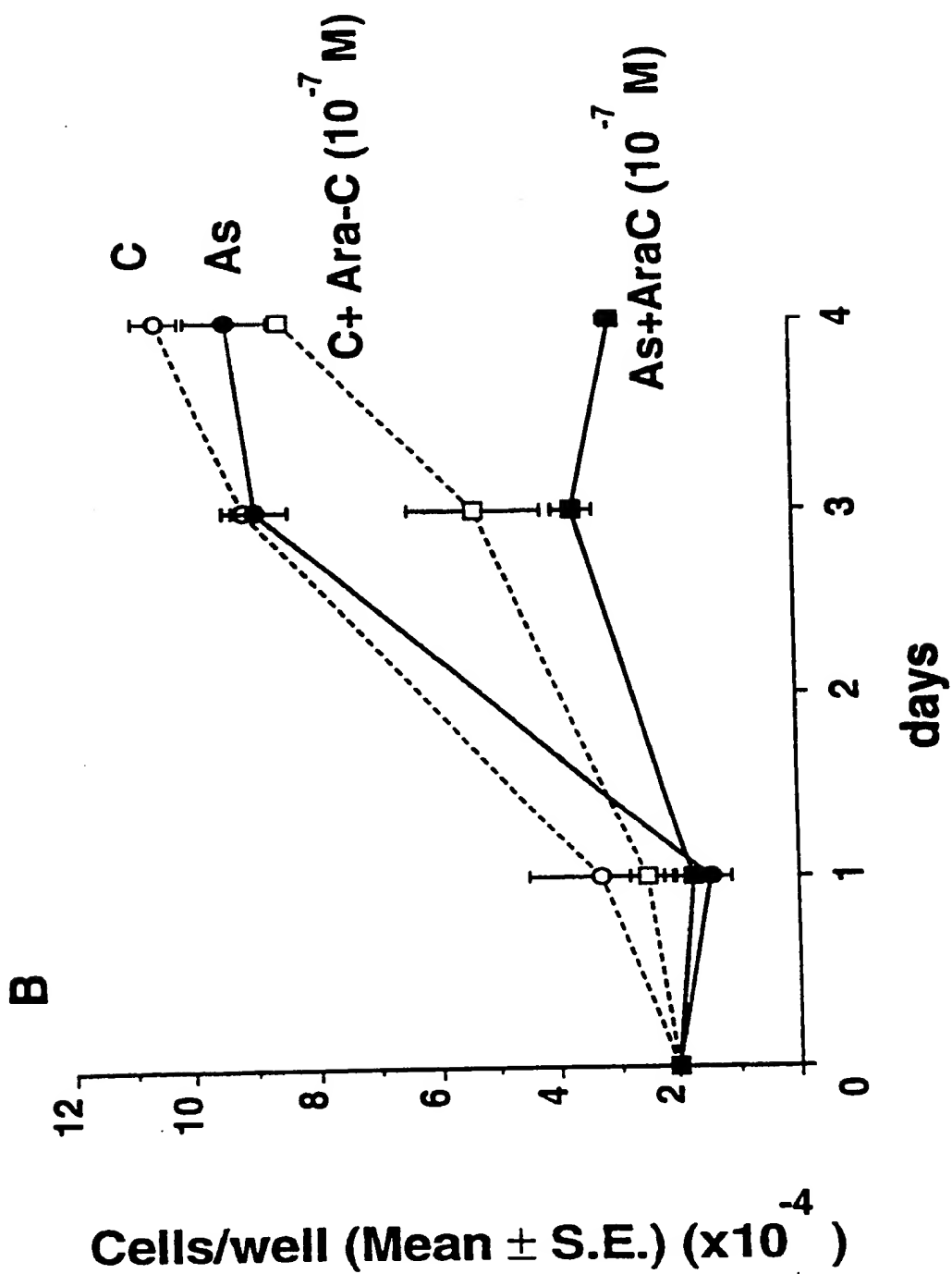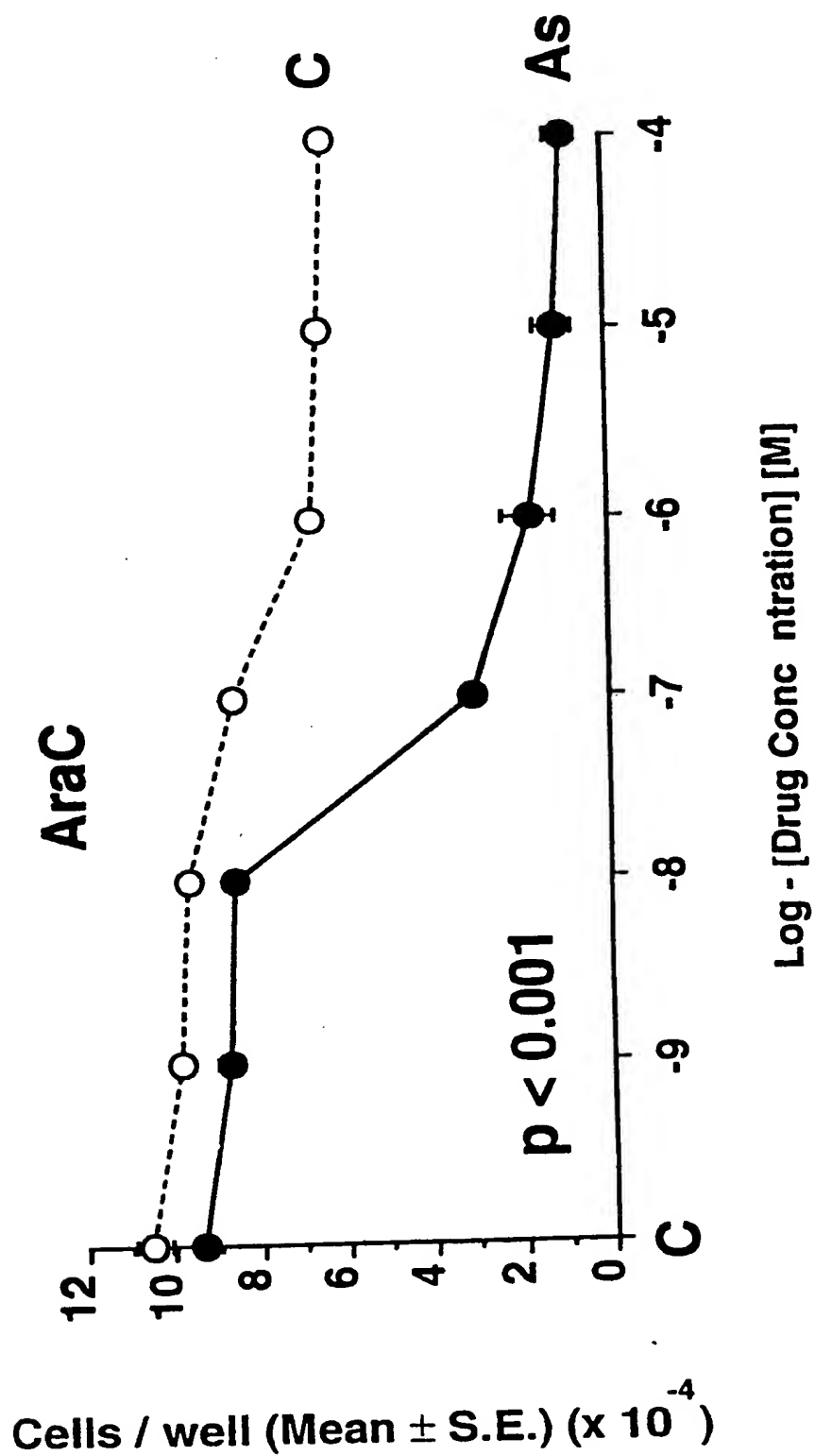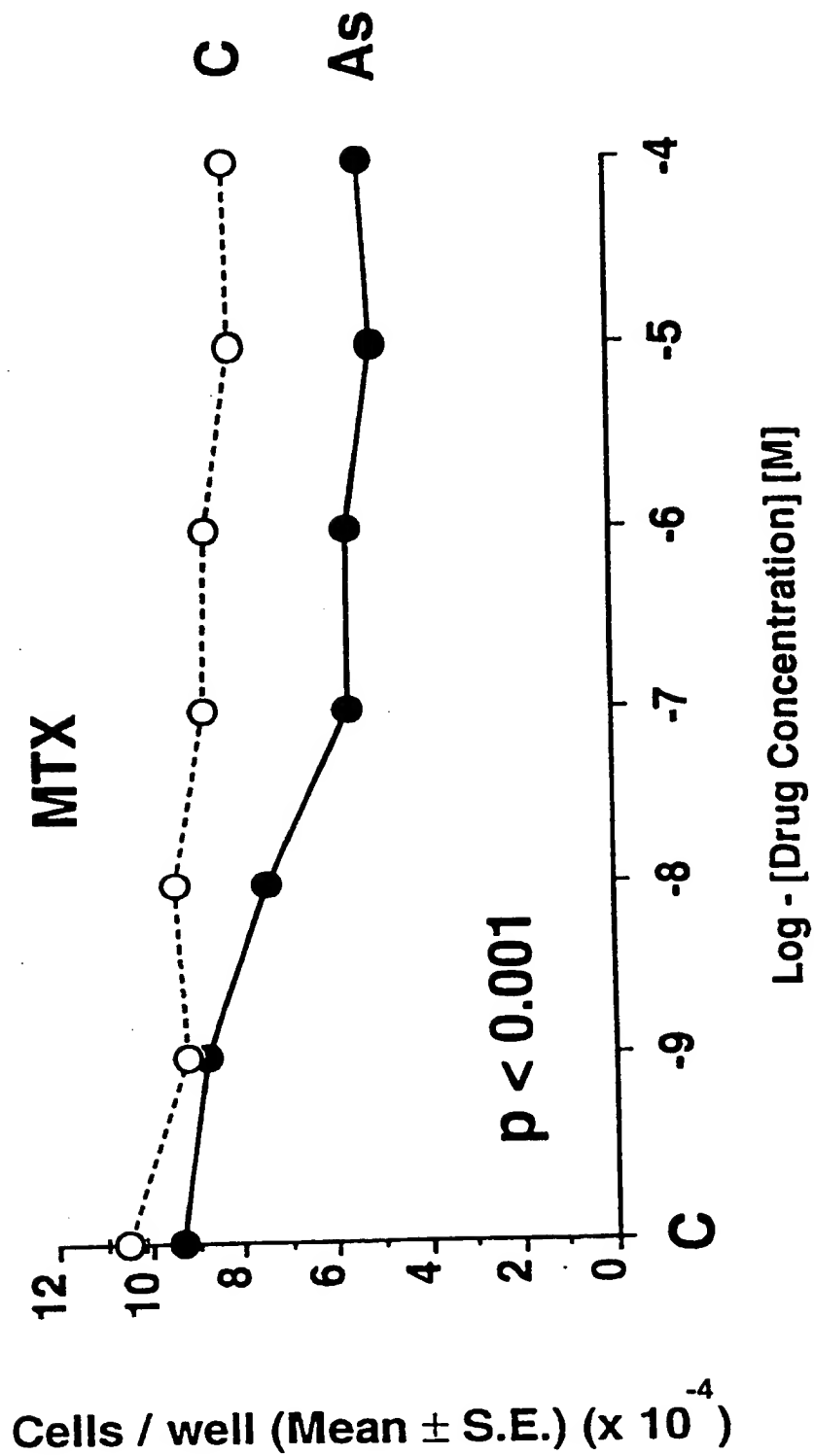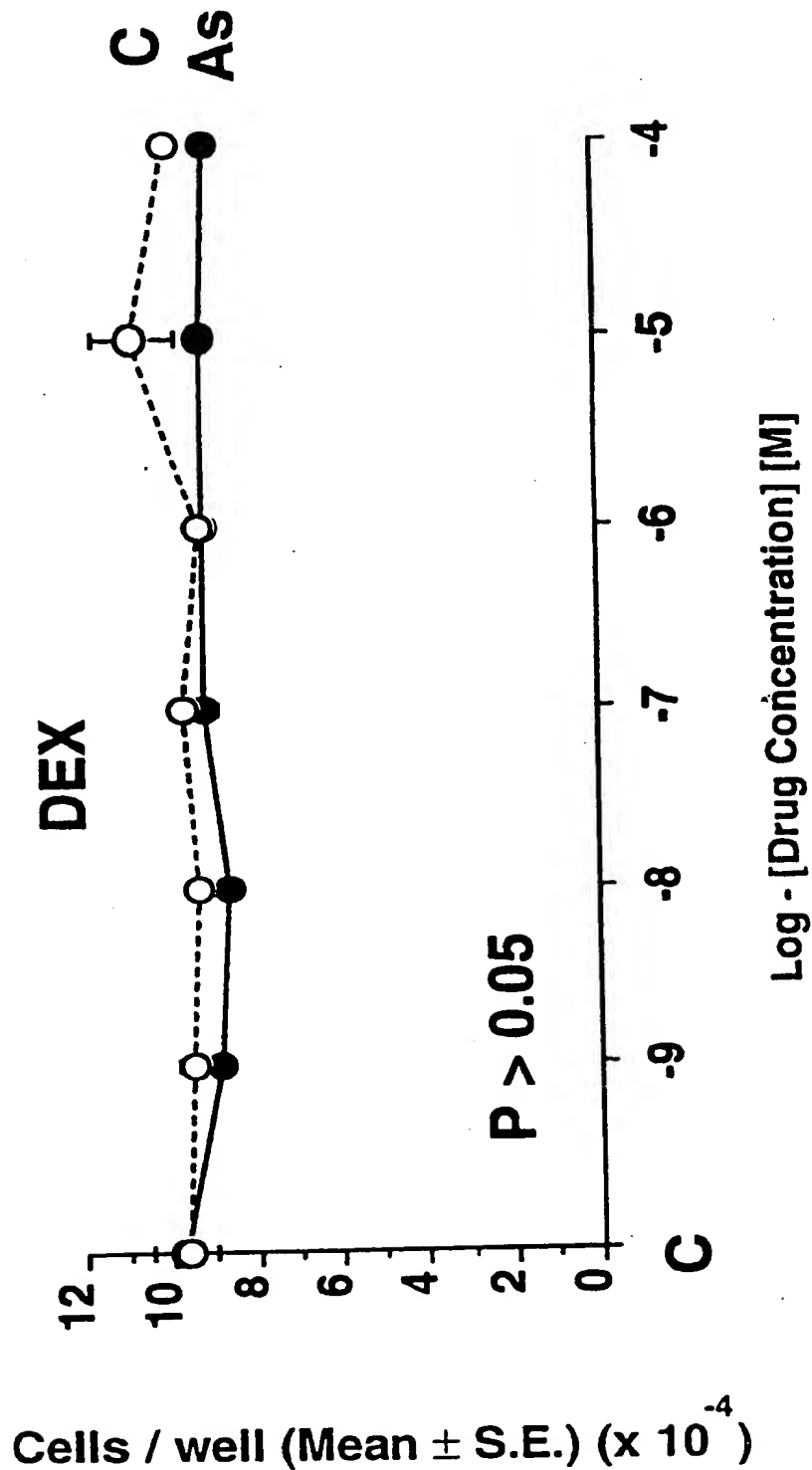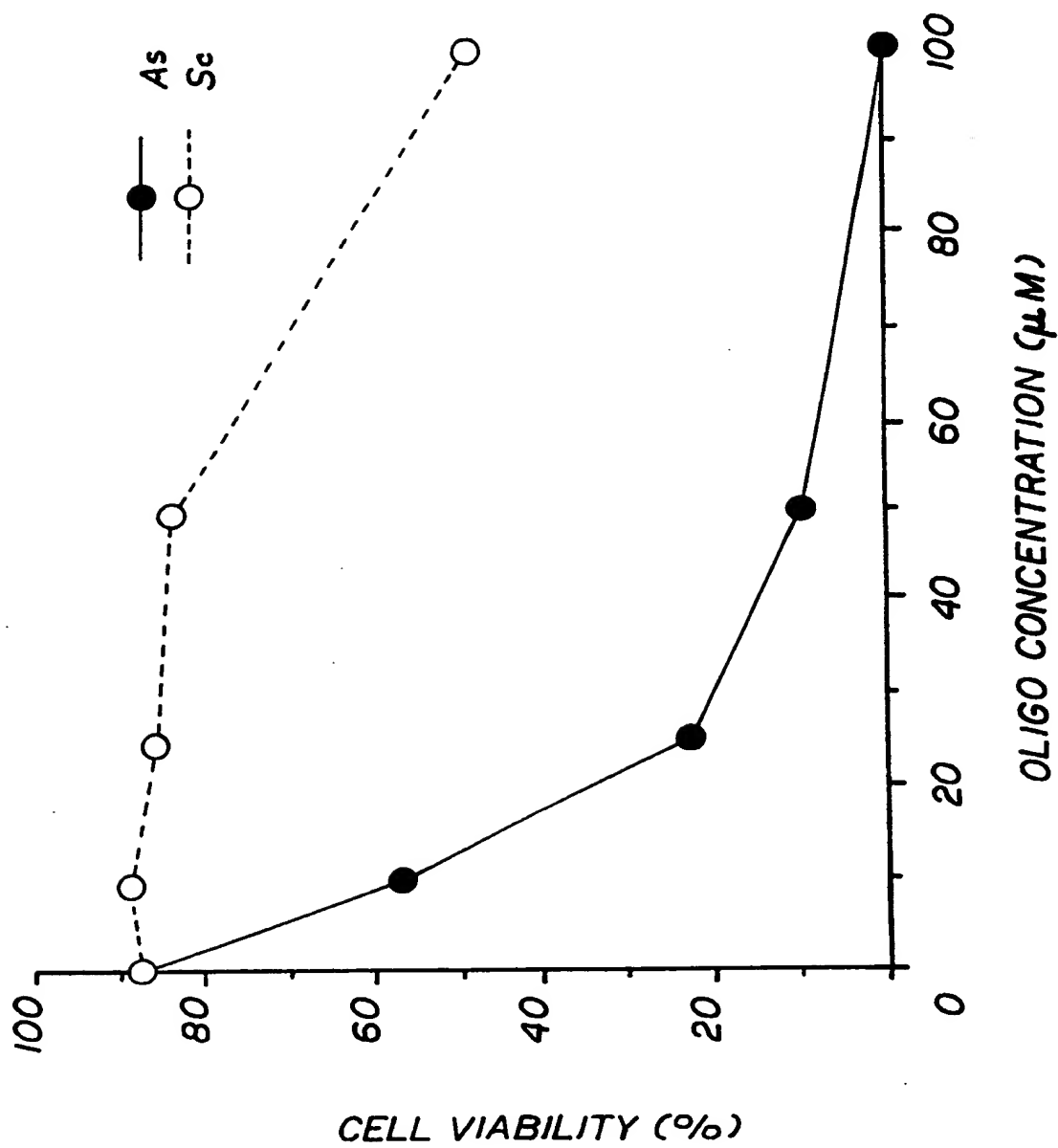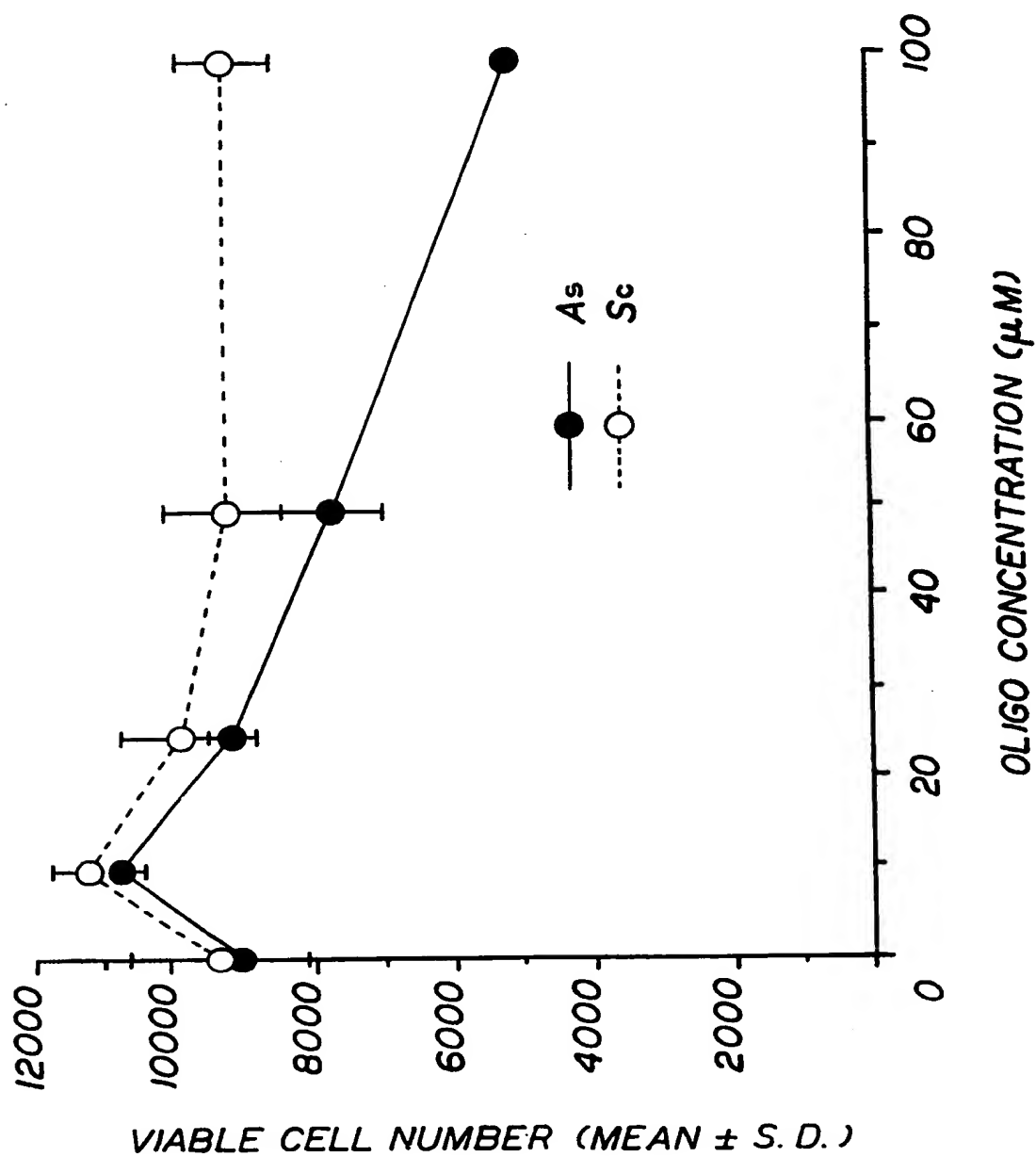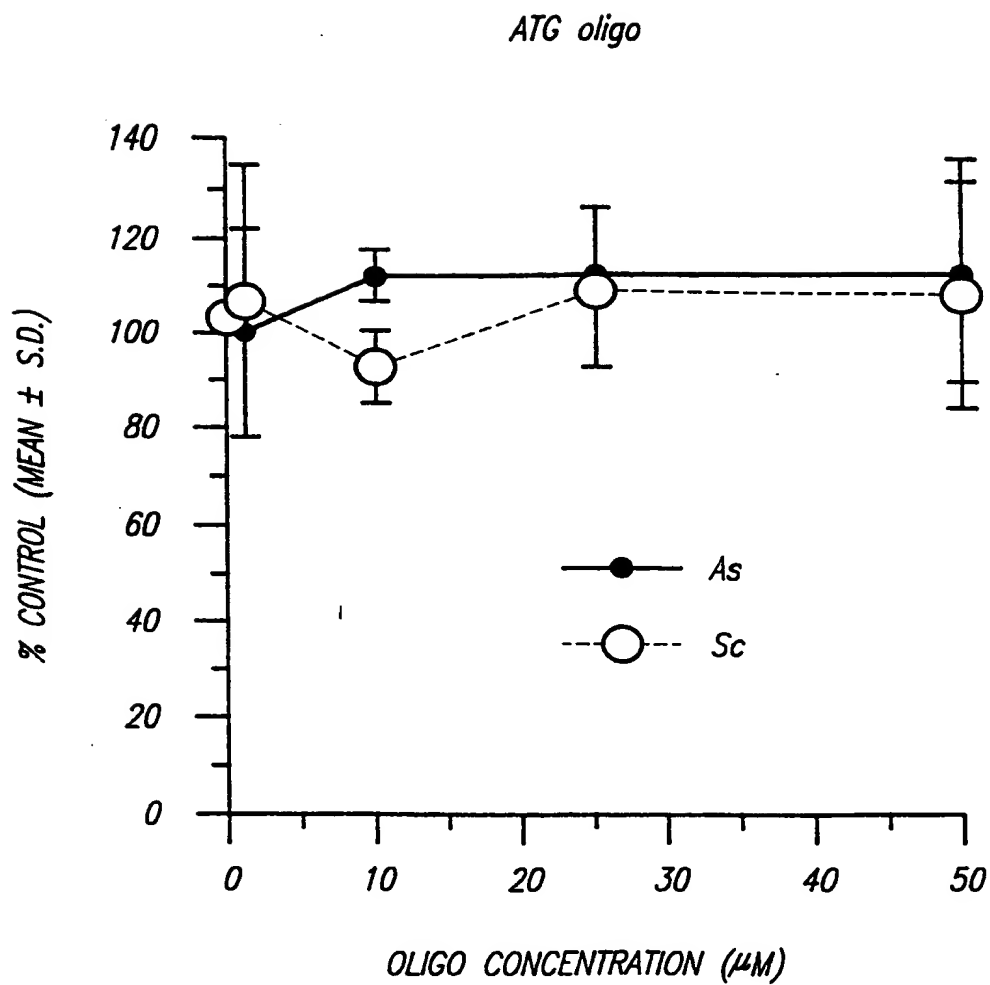
As–18′mer
5′–TCTCCCAGCGTGCGCCAT–3′
Sc–18′mer
5′–TGCACTCACGCTCGGCCT–3′

FIG. 13(b)
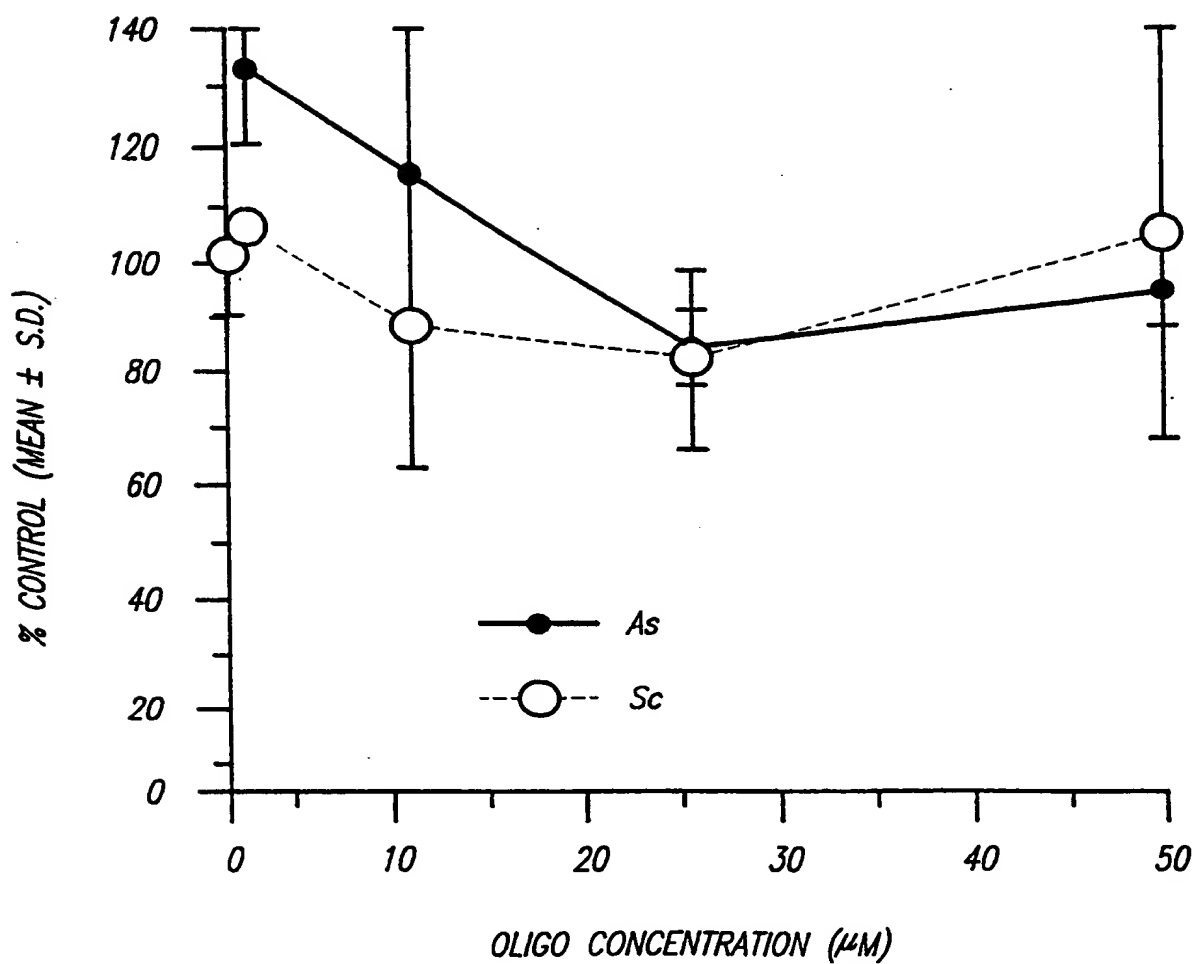
DScore 23 oligo
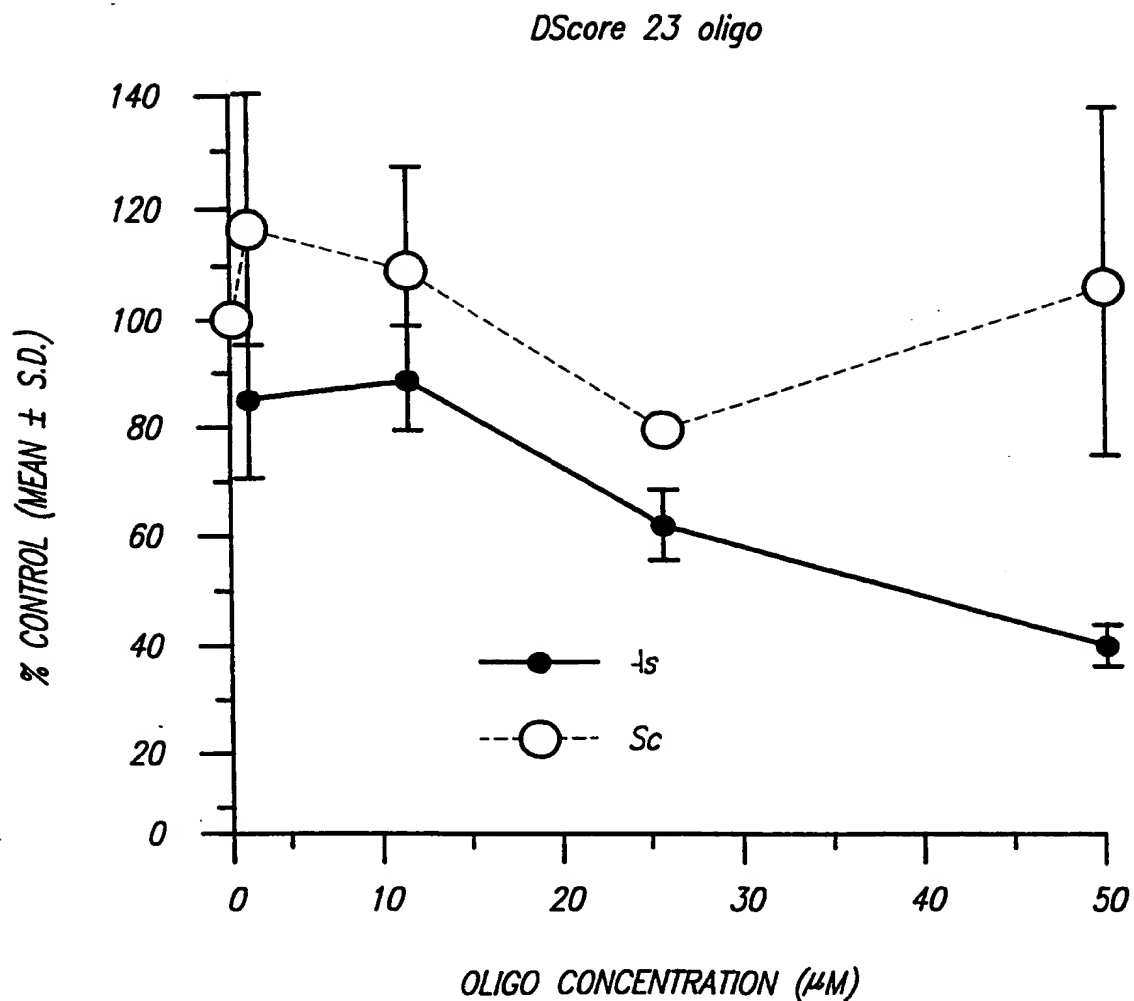
As23
5'-GCGCGGCGGGCGGGCGGGCA-3'
Sc23
5'-GGGCGGAGGCCGGCCGGCGG-3'

FIG. 13(c)

DScore 72

As72
5′-AGCGGCGGCGGCGGCAGCGC-3′
Sc72
5′-GGGCCGGGAAGGGCGCCCGC-3′

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A61K 48/00
US CL   :514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, bcl-2, antisense, anticode, apoptosis, tumor cells, therapeutic agent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Anti-Cancer Drug Design, Volume 2, issued 1988, Paoletti, "Anti-sense oligonucleotides as potential antitumour agents: prospective views and preliminary results", pages 325-331, see entire article.	1-40
Y	Proceedings of the National Academy of Sciences USA, Volume 83, issued July 1986, Tsujimoto et al., "Analysis of the structure, transcripts and protein products of *bcl-2*, the gene involved in human follicular lymphoma", pages 5214-5218, see entire article.	1-40
Y	Cancer Research, Volume 48, issued 15 May 1988, Stein et al., "Oligodeoxynucleotides as inhibitors of gene expression: A review", pages 2659-2668, see entire article.	1-40

☒ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 NOVEMBER 1994	12 JAN 1995

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F rm PCT/ISA/210 (second sheet)(July 1992)*

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Research, Volume 50, issued 15 October 1990, Reed et al., "Antisense-mediated inhibition of *BCL2* protooncogene expression and leukemic cell growth and survival: Comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides", pages 6565-6570, see entire article.	1-40
Y	Cell, Volume 67, issued 29 November 1991, Strasser et al., "*bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship", pages 889-899, see entire article.	1-40
Y	Chemical Reviews, Volume 90, No. 4, issued June 1990, Uhlmann et al., "Antisense oligonucleotides: A new therapeutic principle", pages 544-584, see entire article.	1-40
Y	US, A, 5,098,890 (GEWIRTZ ET AL.) 24 March 1992, see entire document.	1-40